

**Investigation of the Interleukin-1 $\beta$  Converting Enzyme  
- Solid Phase Peptide Synthesis Studies**

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To my parents,  
Margaret & Bruce Urquhart



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## Abstract

An investigation of the substrate requirements of the interleukin-1 $\beta$  (IL-1 $\beta$ ) converting enzyme (ICE) has been carried out. Solid phase peptide synthesis has been used to synthesise sequences from precursor IL-1 $\beta$ , a cowpox inhibitor crmA, and the pro region of ICE. The peptides have been assayed to ascertain their inhibitory properties.

In the course of this work an attempt has been made to establish a simple way of introducing  $\beta$ -turns into peptides. This has been carried out by the formation of a disulphide bridged species in which cystine formation was facilitated by the use of the turn-inducing 'ProD.Val' moiety, and by the introduction of an unnatural amino acid, *cis*-3-aminocyclopentane-1-carboxylic acid (ACPC).

The  $\beta$ -turns developed in the ICE work have been introduced into a peptide containing a discontinuous epitope of gp120, and have been found, in this instance, to be interchangeable with one another, and with the  $\gamma$ -turn Cys-Val-Cys.



## Abbreviations

<b>AAA</b>	amino acid analysis
<b>ACPC</b>	<i>cis</i> -3-aminocyclopentane-1-carboxylic acid
<b>AE</b>	activated ester
<b>AMC</b>	7-amino-4-methylcoumarin
<b>Boc</b>	<i>t</i> -butyloxycarbonyl
<b>CD</b>	circular dichroism
<b>cDNA</b>	complementary deoxyribonucleic acid
<b>CHAPS</b>	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate
<b>crmA</b>	cytokine response modifier A
<b>DCC</b>	N,N'-dicyclohexylcarbodiimide
<b>DCM</b>	dichloromethane
<b>DIC</b>	N,N'-diisopropylcarbodiimide
<b>DIEA</b>	diisopropylethylamine
<b>DMAP</b>	4-dimethylaminopyridine
<b>DMF</b>	N,N-dimethylformamide
<b>DMSO</b>	dimethylsulphoxide
<b>DQ COSY</b>	double quantum filtered correlation spectroscopy
<b>DTNB</b>	5,5'-dithiobis(2-nitrobenzoic acid)
<b>DTT</b>	dithiothreitol
<b>EDT</b>	ethanedithiol
<b>EDTA</b>	ethylenediaminetetracetic acid
<b>EI</b>	electron ionization
<b>FAB</b>	fast atom bombardment
<b>Fmoc</b>	9-fluorenylmethyloxycarbonyl
<b>HCMV</b>	human cytomegalovirus
<b>HEPES</b>	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
<b>HOAt</b>	1-hydroxy-7-azabenzotriazole
<b>HOBt</b>	1-hydroxybenzotriazole
<b>HOCt</b>	ethyl-1-hydroxy-1H-1,2,3-triazole-4-carboxylate
<b>HPLC</b>	high performance liquid chromatography
<b>ICE</b>	interleukin-1 $\beta$ converting enzyme
<b>Ig</b>	immunoglobulin
<b>IL</b>	interleukin
<b>IL-1R</b>	interleukin-1 receptor
<b>IL-1ra</b>	interleukin-1 receptor antagonist
<b>mRNA</b>	messenger ribonucleic acid
<b>NOESY</b>	nuclear Overhauser effect spectroscopy
<b>NTB</b>	nitrothiobenzoate
<b>PGC</b>	porous graphitised carbon
<b>proIL-1<math>\beta</math></b>	precursor interleukin-1 $\beta$
<b>RA</b>	rheumatoid arthritis
<b>RSL</b>	reactive site loop
<b>R<sub>t</sub></b>	retention time
<b>SA</b>	symmetrical anhydride



<b>SPPS</b>	solid phase peptide synthesis
<b>TOCSY</b>	total correlation spectroscopy
<b>TOF</b>	time of flight
<b>TbFmoc</b>	17-tetrabenzo[ <i>a, c, g, i</i> ]fluorenylmethoxycarbonyl
<b>TFA</b>	trifluoroacetic acid
<b>TFE</b>	2,2,2-trifluoroethanol
<b>THF</b>	tetrahydrofuran
<b>TMS</b>	trimethylsilane
<b>TMSBr</b>	trimethylsilyl bromide
<b>TNF</b>	tumour necrosis factor



**The Naturally Occurring Amino Acids**  
**-their 3 & 1 letter codes**

<b>Amino Acid</b>	<b>3 letter code</b>	<b>1 letter code</b>
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V



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# 1. Introduction

## 1.1 Interleukin-1 $\beta$

The interleukins are a subset of the cytokines, protein hormones which mediate and regulate the immune and inflammatory responses. The cytokines are a diverse group which nonetheless share a number of characteristics. The same biological effects can often be attributed to several cytokines, despite the fact that they bind to different receptors, and additive or synergistic effects are often seen when two or more cytokines are present. For example, despite having distinct receptors, interleukin-1 (IL-1) and tumour necrosis factor (TNF) have remarkably similar biological properties, and when used together in experimental studies the observed effect often exceeds the additive effect of the individual hormones working alone<sup>1</sup>. Cytokines often regulate the production of other cytokines and cytokine receptors: for example, IL-1 production is up-regulated both by itself and by transforming growth factor (TGF)- $\beta$ <sup>2</sup>, but down-regulates its own receptor<sup>3</sup>.

### 1.1.1 Biological Effects of IL-1

The term IL-1 encompasses two distinct gene products, IL-1 $\alpha$  and IL-1 $\beta$ . Despite showing only 26% sequence homology in humans,<sup>4</sup> they bind to the same receptors and share many biological properties<sup>1</sup>.

IL-1 has both systemic effects, being fever- and sleep-inducing, and causing appetite suppression, and local autocrine/paracrine effects. Its immunostimulatory properties include the augmentation of T, B and natural killer cell responses. IL-1 is also proinflammatory. It induces endothelial cells to release the prostaglandins PGI<sub>2</sub> and PGE<sub>2</sub>, and to increase their expression of the intercellular adhesion molecule

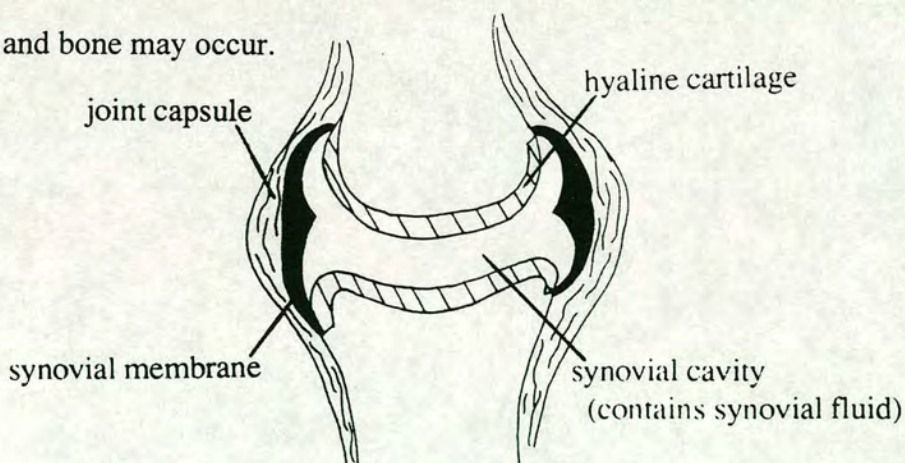


(ICAM)-1, which causes adhesion of neutrophils, monocytes and lymphocytes. IL-1 is, in addition, chemotactic for monocytes, lymphocytes and neutrophils so that cellular infiltration occurs at the site of IL-1 release. This, taken with the fact that IL-1 initiates blood clotting, thus decreasing blood flow, acts to localise tissue inflammation.

IL-1 has both catabolic and anabolic effects. It induces collagenase production and release of metalloproteinases and proteoglycanases, all of which mediate the destruction of bone and cartilage. However, it also causes angiogenesis, the migration and proliferation of endothelial cells which results in the formation of new blood vessels, and collagen synthesis.

### 1.1.2 Rheumatoid Arthritis

Around 2% of the world's population suffer from rheumatoid arthritis (RA), with three female sufferers for every male. Whilst RA is associated with systemic effects such as fever and muscle wasting, it is primarily a disease of the synovial joints (Fig. 1.1). In an RA patient these joints are swollen and painful, and destruction of cartilage and bone may occur.



**Fig. 1.1. A synovial joint**

The pathology of RA suggests that IL-1 may be an important mediator of the disease<sup>5</sup>, and the presence of IL-1 in the synovial fluid of rheumatoid joints is well



established. It has been shown that mononuclear cells from RA patients release IL-1 $\beta$  far more rapidly in response to antigenic challenge than do cells from healthy controls<sup>7</sup>. Administration of murine recombinant IL-1 to mice during the chronic phase of antigen-induced arthritis results in a flare-up of the smouldering inflammation in the joints that is analogous with the flare-ups seen in human RA<sup>6</sup>, and blocking the action of IL-1 reduces bacterial-cell-wall-induced arthritis in rats<sup>8</sup>.

Many of the symptoms of RA could be directly or indirectly related to the presence of IL-1. The rheumatoid synovial lining is characterised by its heavy infiltration by inflammatory cells such as lymphocytes and macrophages (IL-1 is chemotactic for such cells) and the growth of new blood vessels (angiogenesis is stimulated by IL-1<sup>9</sup>). The pain associated with rheumatoid joints is thought to be due to the PGE<sub>2</sub> found in the synovial fluid; as previously noted, IL-1 induces prostaglandin production in endothelial cells. It has been shown that IL-1 stimulates osteoclast recruitment by a mechanism involving prostaglandins, probably PGE<sub>2</sub><sup>9</sup>. Osteoclasts break down the calcified intercellular structure of bone. In addition, IL-1 regulates the production of matrix metalloproteinases such as collagenase and stromelysin<sup>10</sup>, which in turn regulate the breakdown of cartilage. It is interesting to note that IL-1 has been implicated in post-menopausal bone loss leading to osteoporosis<sup>11</sup>.

It has been demonstrated that the predominant source of IL-1 $\beta$  in the synovial tissue of RA patients is CD14 positive macrophages<sup>12</sup>. Whilst not all the CD14 positive macrophages present secrete IL-1 $\beta$ , those doing so are generally found in pairs or clusters, a finding consistent with the fact that IL-1 induces production of further IL-1.

It should be emphasised that, whilst IL-1 may play a key role in the progression of RA, it is not the initial cause. IL-1 is produced in response to disease, and



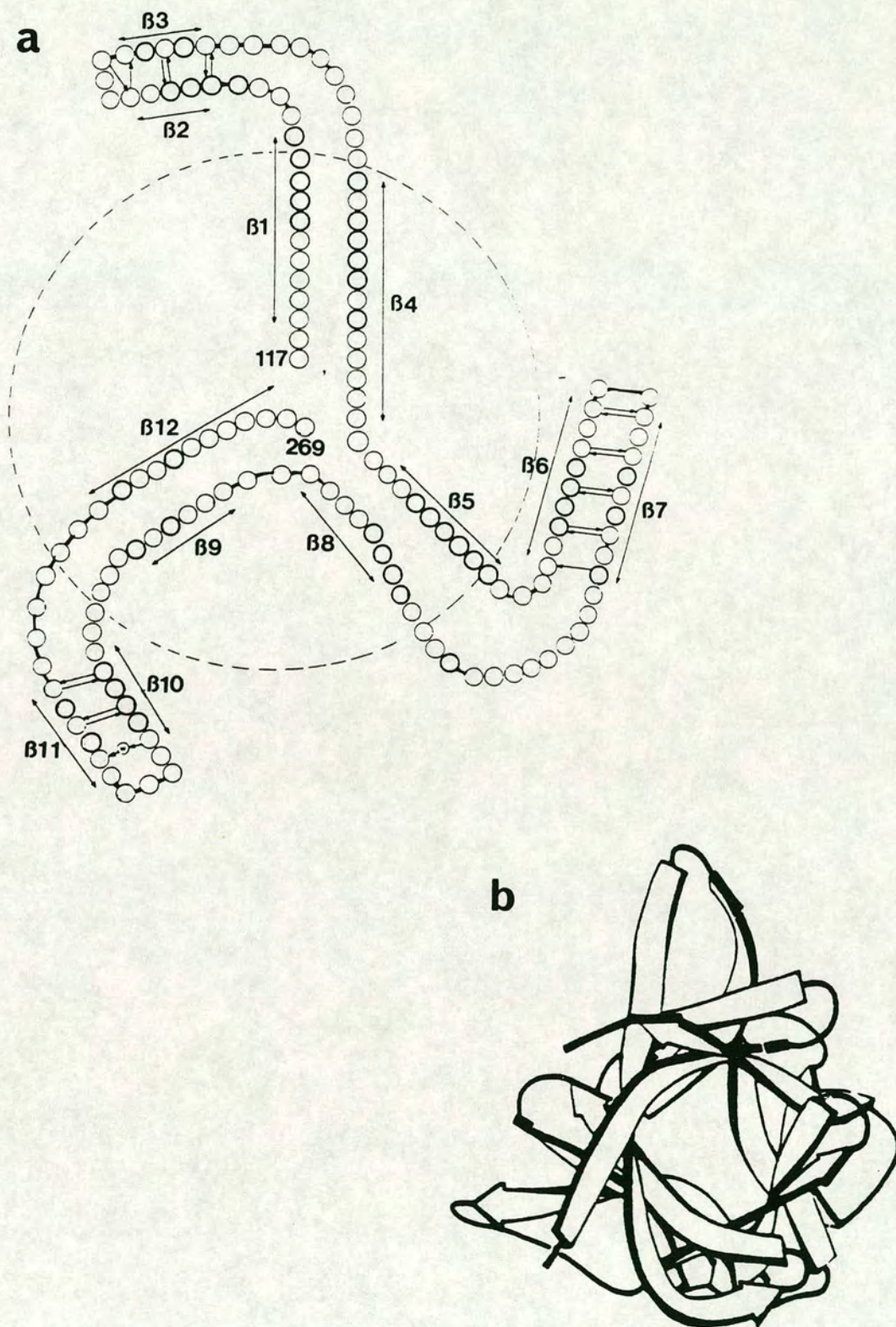
subsequently acts as a mediator of tissue destruction. The body produces natural inhibitors of IL-1 activity which may be co-induced with IL-1 itself. It seems likely that, in autoimmune diseases such as RA, there is an imbalance between IL-1 and its inhibitors, so that IL-1 is not down-regulated effectively and an abnormal inflammatory response develops<sup>13</sup>.

### **1.1.3 IL-1: Two Gene Products - $\alpha$ and $\beta$**

IL-1 $\alpha$  and  $\beta$  are both produced as 31kDa propeptides which can undergo proteolytic processing to mature 17kDa products. Despite having relatively low sequence homology (26%), the mature forms adopt the same tertiary structure, a structure which is shared with soybean trypsin inhibitor and fibroblast growth factor (FGF); it has been dubbed the 'IL-1 fold'<sup>14</sup>. The gross structure is that of a barrel, open at one end. The barrel is constructed from twelve anti-parallel  $\beta$ -strands, three pairs forming the 'struts' and the remaining three covering the 'closed' end. The structure has three-fold rotational symmetry; it consists of three subunits, each taking the form of three  $\beta$ -strands, a loop and a final  $\beta$ -strand<sup>15</sup> (Fig. 1.2). The inner core of the barrel is lined with hydrophobic residues.

Given their similar structures, it is perhaps not surprising that mature IL-1 $\alpha$  and  $\beta$  bind to the same receptors with similar affinities. However, whilst proIL-1 $\alpha$  also binds to the receptor and elicits a biological response, proIL-1 $\beta$  does not bind<sup>16</sup>. This suggests a difference in the structures of the  $\alpha$ - and  $\beta$ -forms such that the cleaved portion of proIL-1 $\beta$  sterically inhibits interaction with the receptor, whilst that of proIL-1 $\alpha$  does not. Inspection of the crystal structures of mature IL-1 $\alpha$  and  $\beta$  shows that, whilst in both cases the N and C termini are situated at the open end of the barrel, in IL-1 $\alpha$  they are at opposite sides of the 'rim' whereas in IL-1 $\beta$  they are close together<sup>14,17</sup>.





**Fig. 1.2. The structure of IL-1 $\beta$**

**a. Schematic of IL-1 $\beta$  showing the pseudo-3-fold symmetry**

**b. Ribbon diagram of IL-1 $\beta$ , viewed from the open end of the barrel**

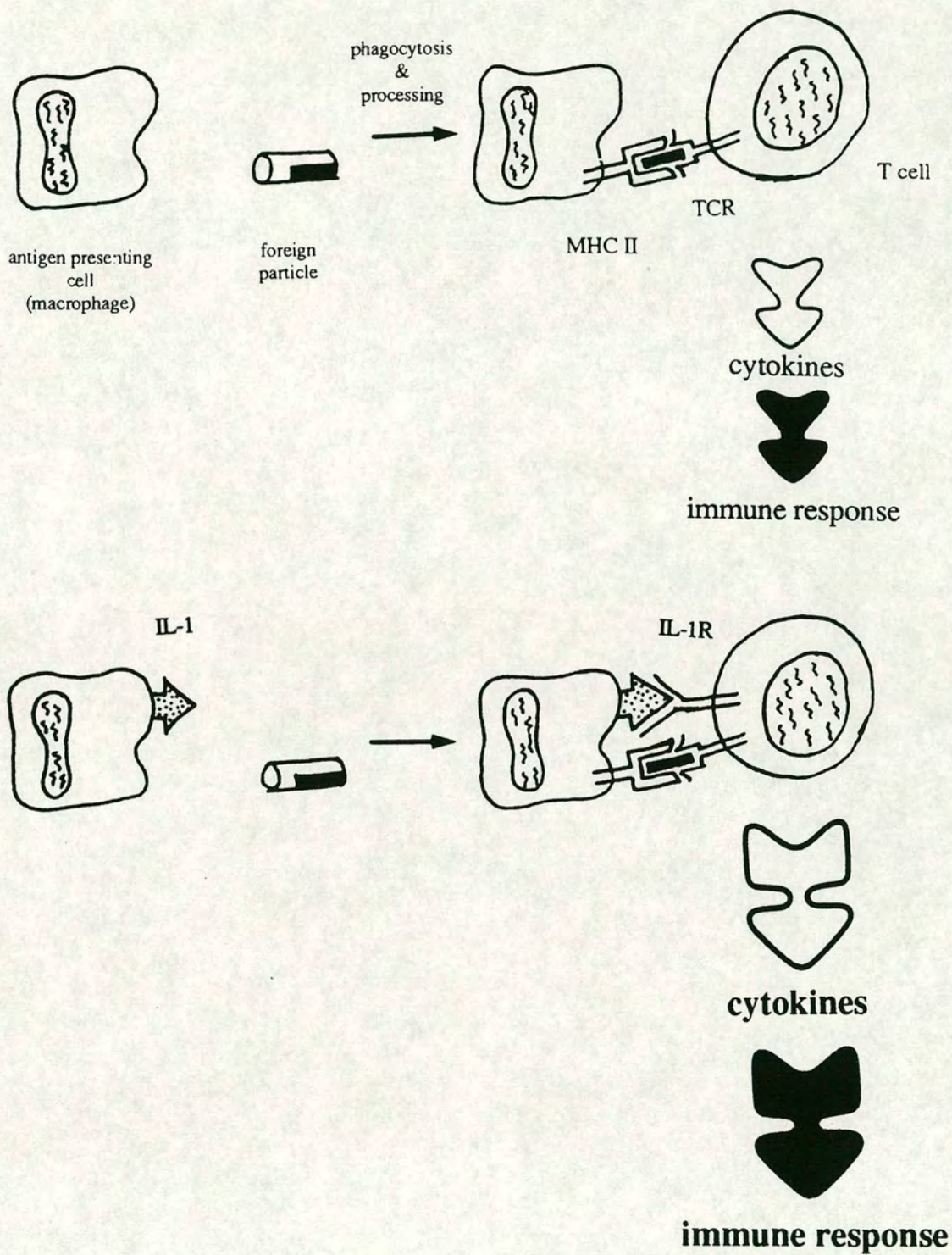


Since the biological activities of IL-1 $\alpha$  and  $\beta$  are so similar, the reason for the maintenance of two separate gene products is uncertain. However, insight may be gleaned from differences in the kinetics of secretion of the two forms. IL-1 $\beta$  has a much shorter intracellular half life than IL-1 $\alpha$  (2.5 hours compared with 15 hours), and in addition is released much sooner after stimulation of the cell<sup>18</sup>. In general, IL-1 $\alpha$  tends to remain associated with the cell, with IL-1 $\beta$  being the main secreted form. IL-1 activity is expressed on the cell surfaces of macrophages and is associated with a protein of approximately 30kDa molecular weight, suggesting a membrane-bound form of proIL-1 $\alpha$ . It has been shown that transfection of fibroblasts with proIL-1 $\alpha$  cDNA results in the expression of membrane-associated IL-1 activity, whereas transfection with proIL-1 $\beta$  cDNA does not<sup>19</sup>. It has been suggested that this membrane-bound proIL-1 $\alpha$  could interact with T cell IL-1 receptors during antigen presentation by the macrophage thus up-regulating the T cell response (Fig. 1.3). Therefore it appears that the cell-associated IL-1 $\alpha$  may play a larger part in immunostimulation, whilst the freely moving, extracellular IL-1 $\beta$  may be more important in regulating the inflammatory response.

IL-1 $\alpha$	IL-1 $\beta$
Propeptide 31kDa Mature form 17kDa	Propeptide 31kDa Mature form 17kDa
Adopts the 'IL-1 fold'	Adopts the 'IL-1 fold'
Binds to type I and type II receptors	Binds to type I and type II receptors
Precursor is biologically active	Precursor is <u>not</u> biologically active
Mainly cell-associated	Mainly extracellular
Immunostimulatory	Proinflammatory

**Table 1.1. Similarities and differences between IL-1 $\alpha$  &  $\beta$**





**Fig. 1.3. Cartoon showing the proposed interaction of cell surface IL-1 with the T cell receptor, resulting in up-regulation of the immune response**



#### 1.1.4 The IL-1 Receptor and its Interaction with IL-1 $\beta$

Two IL-1 receptors have been described, designated type I and type II. Although their extracellular structures are very similar, the type II receptor has a very short cytoplasmic domain of only 29 amino acids. This has led to doubts as to whether IL-1R II is a true receptor, capable of transmitting signals across the cell membrane. Whilst a chimeric receptor composed of the extracellular and transmembrane regions of type II and the cytoplasmic domain of type I has been shown to mediate IL-1 effects<sup>20</sup>, there is evidence that the type II receptor itself does not transduce signals. It has been suggested by Colotta *et al*<sup>21</sup> that the type II 'receptor' is, rather, a decoy trap for IL-1, thus assisting in the regulation of this cytokine.

The type I receptor is found predominantly on T cells, fibroblasts and macrophages. However, since very low levels of receptor occupancy are required for signalling to take place, it is possible that other cell types that respond to IL-1 carry IL-1R I in undetectably small amounts. It is interesting that, whilst low levels of IL-1 result in a biological response, at higher IL-1 levels the IL-1R/cytokine complex is observed to undergo endocytosis. Internalised IL-1 is thought to become associated with the nucleus, and it is possible that interaction of IL-1 with nuclear receptors may be important for some of its biological effects<sup>22</sup>.

The type I receptor consists of three distinct regions<sup>23</sup>. The transmembrane region is a single strand, 21 amino acids long, composed predominantly of hydrophobic residues. The extracellular region is 319 amino acids long and is organised into three immunoglobulin(Ig)-like domains. Such domains consist of a pair of  $\beta$ -strands, pinned together by a disulphide bond. The cysteines and certain other key residues important for structure formation are highly conserved in all Ig-like molecules, but typically minimal amino acid similarity is seen at other positions. This structural



organisation (shared with IL-1R II) makes the IL-1R a member of the immunoglobulin superfamily. The platelet-derived growth factor (PDGF) receptor is also a member of this class. The extracellular region contains seven potential N-linked glycosylation sites, and the IL-1R I is known to be glycosylated on Asn. The cytoplasmic domain is 217 amino acids long and shows no significant similarity to known sequences. In particular, it has no regions typical of tyrosine kinases as found in other Ig-like receptors. The signalling mechanism is thus unclear, but may involve a novel, as yet uncharacterised, protein kinase<sup>24</sup>.

Various studies have attempted, by the use of site-directed mutagenesis and neutralising antibodies, to define the regions of IL-1 $\beta$  that are important for IL-1R I binding. Residues that have been identified as important are Arg-120, Leu-122, Phe-162, Ile-172, Lys-208, Lys-209, Lys-210, Arg-214, Lys-219 and Glu-221<sup>17,25</sup>. All these residues are situated on the rim of the open end of the 'IL-1 fold barrel'. Further residues have been identified which, whilst apparently having a minimal effect on the binding energy, are important for biological activity. Analysis of such results has led Clore *et al*<sup>26</sup> to propose three distinct regions of IL-1 $\beta$ , each of which interacts with one of the Ig-like domains of the IL-1R. The first region, defined by the residues specified above, is thought to contribute most to the binding energy, with the remaining two sites playing a part in signal transduction.

## **1.1.5 Blocking IL-1**

### **1.1.5.1 IL-1 Binding Proteins**

There are conceivably two ways in which proteins and peptides can prevent interaction of IL-1 with its receptor and hence modulate its biological effects; they may interact either with IL-1 itself or with the receptor. Various proteins of the first



type are known. The first of these, uromodulin, is an 85kDa glycoprotein found in the urine of pregnant women and produced in the kidneys. Its activity depends on correct glycosylation and it binds both IL-1 and TNF. Its role appears to be the removal of IL-1 from the blood stream<sup>27, 28</sup>.

Symons *et al*<sup>29</sup> have described an IL-1 binding protein found in the supernatant of stimulated Raji B cells. They suggest that this is a proteolytically cleaved form of the IL-1R. Interestingly, this soluble IL-1R binds both precursor and mature IL-1 $\beta$ , but not IL-1 $\alpha$ . A similar IL-1 $\beta$ -specific binding protein has been found to be secreted by vaccinia and cowpox viruses. The 33kDa B15R gene product is a member of the immunoglobulin superfamily and shows significant homology to the IL-1R II. The B15R gene is thought to be under the control of an early promoter, meaning that expression may be rapid and abundant after infection. Thus such gene products help to knock out the host defence mechanisms, giving the virus an advantage in the early stages of infection<sup>30,31</sup>.

It has been reported that a synthetic peptide, amino acids 86-93 of the extracellular domain of the human IL-1R I, binds to both IL-1 $\alpha$  and  $\beta$ , preventing their interaction with the receptor and hence inhibiting biological activity<sup>42</sup>.

#### **1.1.5.2 The IL-1 Receptor Antagonist**

The second type of IL-1 inhibitor, those that interact with the receptor, is represented by the third member of the IL-1 gene family, the IL-1 receptor antagonist (IL-1ra). IL-1ra was purified from human monocytes by Hannum *et al*<sup>32</sup>. It was obtained as three glycosylation variants of a single protein, of nonglycosylated weight 18kDa; the glycosylated and nonglycosylated forms have the same inhibitory activity. The protein has been shown to be a true antagonist<sup>33</sup>, binding to the receptor but eliciting



no response; the IL-1ra/IL-1R complex is not internalised<sup>34</sup>. IL-1ra has been shown to block the activity of IL-1 *in vitro* and *in vivo*, and to reduce the severity of disease in various animal models<sup>35</sup>, notably to reduce joint swelling by 60% in a rat model of RA<sup>8</sup>.

Comparison of the amino acid sequences of IL-1ra, IL-1 $\alpha$  and IL-1 $\beta$  reveals 18% homology between IL-1ra and IL-1 $\alpha$  and homology of 26% between IL-1ra and IL-1 $\beta$ <sup>33</sup>. Therefore it is unsurprising that IL-1ra appears to adopt the 'IL-1 fold'<sup>36</sup>. However, unlike IL-1 $\alpha$  and  $\beta$  which are secreted from the cell by an uncharacterised pathway, IL-1ra has a classical leader sequence and secretion occurs in the conventional way. Hence IL-1ra exists as glycosylated species, whereas neither IL-1 $\alpha$  nor  $\beta$  is seen in glycosylated forms, despite the existence of potential glycosylation sites. Virtually all the IL-1ra produced by a cell is exported, whereas significant amounts of IL-1 $\alpha$  and  $\beta$  remain cell-associated.

It has been suggested that the severity of certain disease states may be due to an imbalance in the production of IL-1ra and IL-1 $\beta/\alpha$ . The anti-inflammatory cytokine interleukin-4 (IL-4) up-regulates the production of IL-1ra in stimulated monocytes, but down-regulates IL-1 $\alpha$  and  $\beta$ . In an animal model of arthritis, treatment with IL-4 has been shown to increase expression of IL-1ra with an accompanying suppression of joint swelling and tissue destruction<sup>39,40</sup>.

Human cytomegalovirus (HCMV), an important cause of morbidity in immunosuppressed individuals, alters immune function. Monocytes infected with HCMV show altered cell-mediated immune responses. It has been shown that HCMV immediate early (IE) gene products regulate both IL-1 $\beta$  and IL-1ra mRNA, together causing increased release of IL-1ra. Of the two IE gene products, 1 and 2, IE2 up-regulates IL-1ra mRNA expression and protein release, and IE1 down-



regulates it, whilst these genes have the directly opposite effect on IL-1 $\beta$ . Hence immune function can be increased or decreased depending on which gene product predominates in the cell<sup>41</sup>.

Due to the extremely low occupancy (2-15%)<sup>3</sup> of the IL-1R required for a biological response, large molar excesses (100-300 fold) of IL-1ra are generally required to inhibit the action of IL-1<sup>37</sup>. Nonetheless, recombinant IL-1ra has been used in clinical trials for the treatment of rheumatoid arthritis<sup>35</sup>.

### **1.1.6 Processing and Secretion - the IL-1 $\beta$ Converting Enzyme**

All forms of IL-1,  $\alpha$  and  $\beta$ , precursor and mature, are found in the culture supernatant of monocytes; thus processing of the precursor to the mature form is not necessary for secretion<sup>18</sup>. In addition, no mature IL-1 is found within the cell, and the precursor does not undergo processing after secretion. Therefore processing must occur immediately prior to secretion, which occurs via a novel pathway enhanced by an elevation in intracellular calcium ions.

As previously mentioned, whilst IL-1 $\alpha$  and  $\beta$  are very similar structurally and in their biological effects, their secretion kinetics are quite different. It is perhaps unsurprising, therefore, that the two precursors are processed to the mature forms by different enzymes, IL-1 $\alpha$  by calpain, a calcium-dependent protease, and IL-1 $\beta$ , between Asp116 and Ala117 (Fig. 1.4), by the IL-1 $\beta$  converting enzyme (ICE). Since only the mature form of IL-1 $\beta$  is capable of effecting a biological response, ICE is clearly an important therapeutic target, the more so given the high specificity of this enzyme for its substrate.



1	MAEVPKLASE	MMAYYSGNED	DLFFEQDGPK	QMKCSFQDLD	LCPLDGGIQL
51	RISDHHYSKG	FRQAASVVVA	MDKLRKMLVP	CPQTFQENDL	STFFPFIFEE
101	EPIFFDTWDN	EAYVHDAPVR	SLNCTLRDSG	QKSLVMSGPY	ELKALHLQGQ
151	DMEQQVVFSM	SFVQGEESND	KIPVALGLKE	KNLYLSCVLK	DDKPTLQLES
201	VDPKNYPKKK	MEKR FVFNKI	EINN KLEFES	AQFPN WYIST	SQAENMPVFL
251	GGTKGGQDIT	DFTMQFVSS			

			Processing		
			▼		
		APVR	SLNCTLRDSG	QKSLVMSGPY	ELKALHLQGQ
151	DMEQQVVFSM	SFVQGEESND	KIPVALGLKE	KNLYLSCVLK	DDKPTLQLES
201	VDPKNYPKKK	MEKR FVFNKI	EINN KLEFES	AQFPN WYIST	SQAENMPVFL
251	GGTKGGQDIT	DFTMQFVSS			

Asn Glu Ala Tyr Val His Asp | Ala Pro Val Arg Ser Leu Asn

P<sub>7</sub> P<sub>6</sub> P<sub>5</sub> P<sub>4</sub> P<sub>3</sub> P<sub>2</sub> P<sub>1</sub> P<sub>1</sub>' P<sub>2</sub>' P<sub>3</sub>' P<sub>4</sub>' P<sub>5</sub>' P<sub>6</sub>' P<sub>7</sub>'

Fig. 1.4. Sequence and processing site of precursor IL-1β

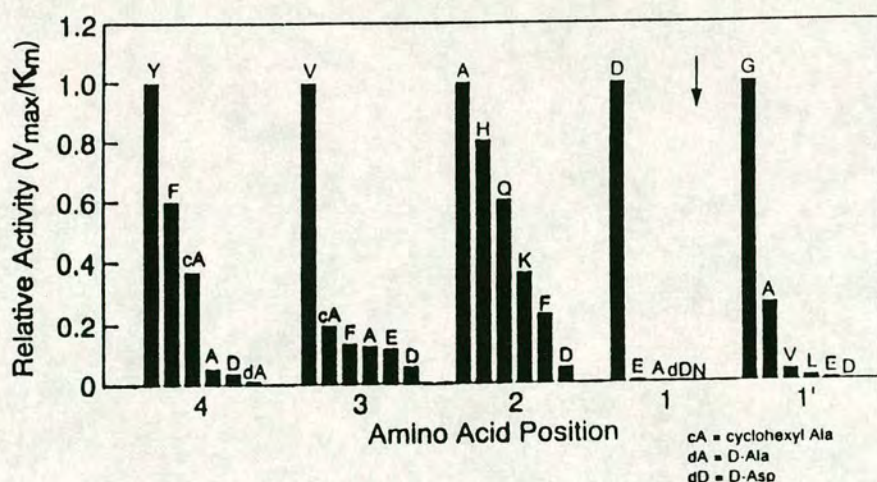


Fig.1.5. Effect of amino acid substitution on ICE activity

ICE activity of a pentapeptide with the indicated substitutions, relative to AcYVADG  
after Miller *et al* <sup>84</sup>

Several groups have investigated the substrate specificity of ICE<sup>43,44,45</sup>. The enzyme has an absolute requirement for an aspartic acid residue adjacent and N-terminal to the scissile bond; no other residue, including glutamic acid, is tolerated at this







bearing no resemblance to other, well-known cysteine proteases such as papain and cathepsin B.

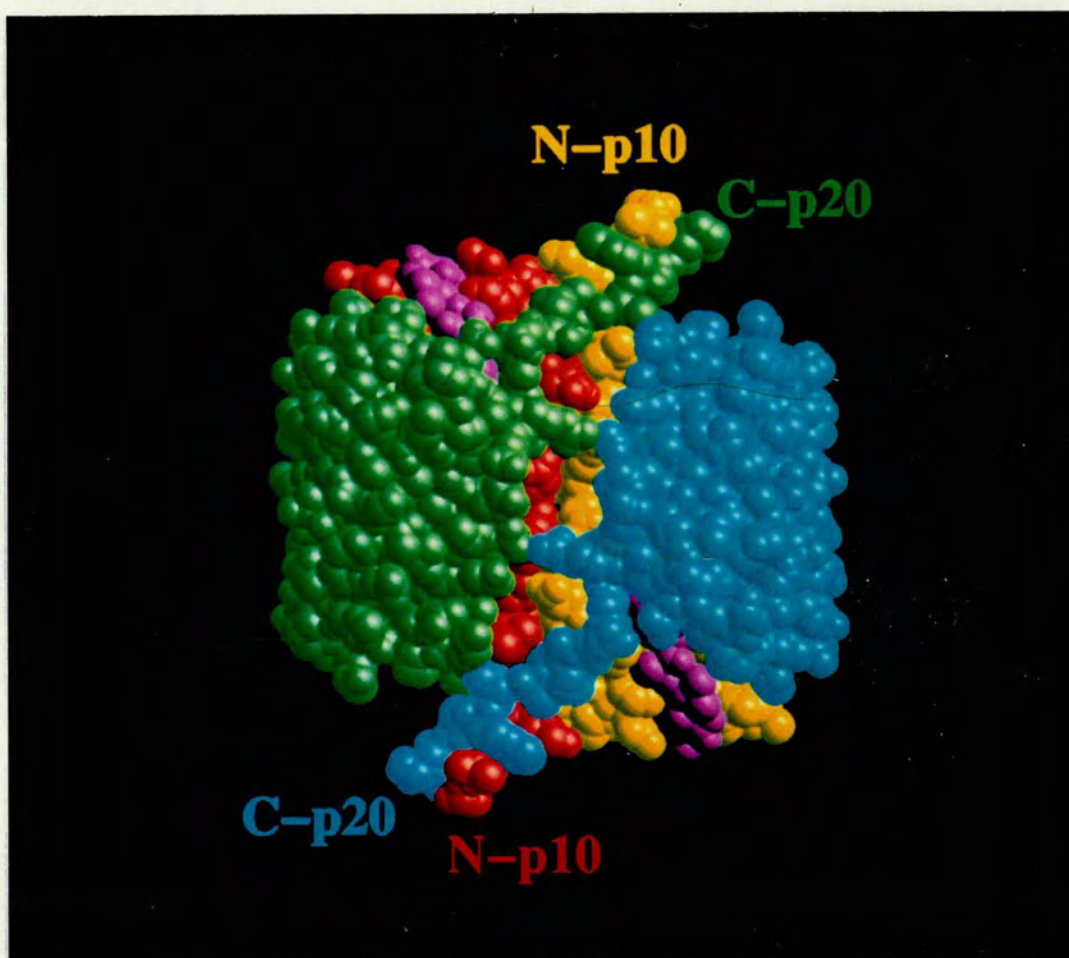
1	MADKVLKEKR	KLFIRSMGEG	TINGLLDELL	QTRVLNKEEM	EKVKRENATV
51	MDKTRALIDS	VIPKGAQACQ	ICITYICEED	SYLAGTLGLS	ADQTSGNYLN
101	MQDSQGVLS	FPAPQAVQDN	PAMPTSSGSE	GNVKLCSLEE	AQRIWKQKSA
151	<u>EIYPIMDKSS</u>	<u>RTRLALIICN</u>	<u>EEFDSIPRRT</u>	<u>GAEVDITGMT</u>	<u>MLLQNLGYSV</u>
201	<u>DVKKNLTASD</u>	<u>MTTELEAFAH</u>	<u>RPEHKTS DST</u>	<u>FLVFMSHGIR</u>	<u>EGICGKKHSE</u>
251	<u>QVPDILQLNA</u>	<u>IFNMLNTKNC</u>	<u>PSLKDKPKVI</u>	<u>IIQACRGDSP</u>	<u>GVVWFKDSVG</u>
301	<u>VSGNLSLPTT</u>	<u>EEFEDDAIKK</u>	<u>AHIEKDFIAF</u>	<u>CSSTPDNVSW</u>	<u>RHPTMGSVFI</u>
351	<u>GRLIEHMQEY</u>	<u>ACSCDVEEIF</u>	<u>RKVRFSFEQP</u>	<u>DGRAQMPSTE</u>	<u>RVTLTRCFYL</u>
401	<u>FPGH</u>				

**Fig. 1.6. The 45kDa proenzyme**  
**p20 and p10 underlined**

At first it was supposed that ICE was a heterodimer composed of a p10 and a p20 subunit; however, from the X-ray crystal data of ICE co-crystallised with the tetrapeptide aldehyde reversible inhibitor AcTyrValAlaAspH<sup>48</sup> and the tetrapeptide chloromethylketone irreversible inhibitor AcTyrValAlaAsp-CMK<sup>85</sup>, it appears that the enzyme is a tetramer composed of a pair of heterodimers (Fig. 1.7). The overall structure is that of two adjacent p10 subunits enfolded by two p20 subunits. That most of the interaction between the heterodimers occurs through p10 is consistent with the fact that p10 is more highly conserved (81%) between human and murine ICE than p20 (59%)<sup>51</sup>. Of the sixteen p10 and six p20 residues located at the tetramer interface, thirteen and four respectively are conserved. It might be supposed that the p20 and p10 units of a heterodimer would originate from the same proenzyme; however, consideration of the p20 C-terminal-p10 N-terminal distances leads to the conclusion that each heterodimer arises from two 45kDa molecules.



Consideration of the proposed active site reveals why both p20 and p10 are required for enzyme activity, as it spans the subunits. The important catalytic residues, Cys285 and His237, are located on p20, whilst p10 residues Val338 and Trp340-Pro343 form the S<sub>2</sub> to S<sub>4</sub> interaction sites. Residues from both p20 and p10, Arg179

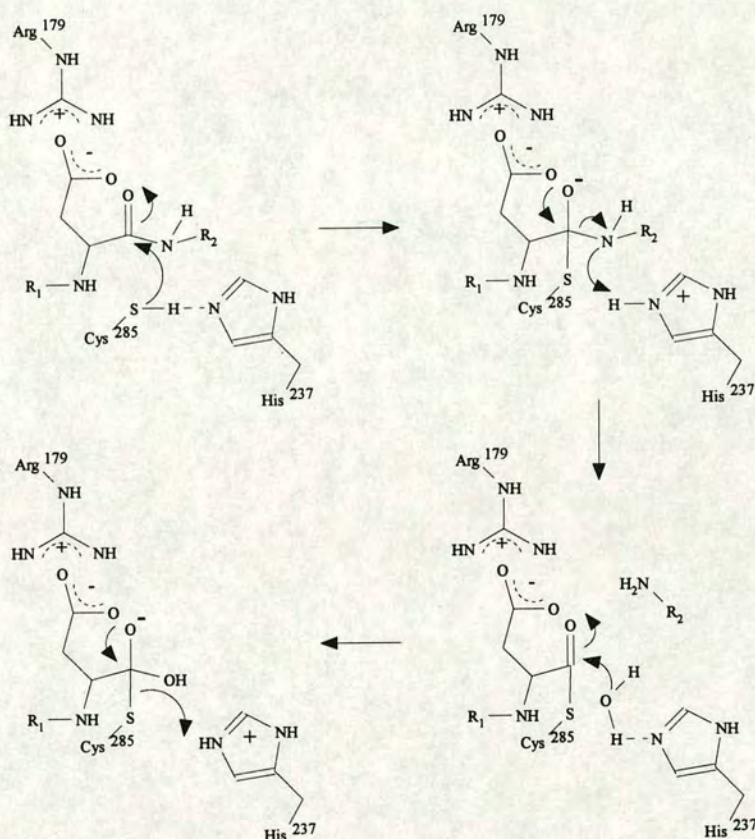


**Fig. 1.7. The structure of ICE**

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and Arg341, are believed to interact with the P<sub>1</sub> Asp, with Arg179 being the more important residue in this respect. The P<sub>2</sub> and P<sub>3</sub> residues of the inhibitor used for co-crystallisation are seen to be exposed to the solvent, explaining the high substitution tolerance at these positions. Based on their observations, Wilson *et al* have proposed a mechanism for the action of ICE (Fig. 1.8).



**Fig. 1.8. Proposed mechanism for ICE**

The cloning of the gene *ced-3* from the nematode *Caenorhabditis elegans* has led to the proposal of a second role for ICE, since it shares significant sequence homology with the protein CED-3<sup>52</sup>. The proteins share 29% amino acid identity overall, with this figure rising to 43% in the most highly conserved regions, 246-360 of CED-3 and 166-287 of ICE. In particular, the pentapeptide QACRG containing the active site Cys is conserved between human and murine ICE and CED-3 of three nematode species, suggesting that CED-3 may also function as a cysteine protease.



The *ced-3* gene is crucial for the induction of programmed cell death (apoptosis), which is vital for normal growth and development. Due to its striking sequence similarity, it has been suggested that ICE may also act as a cell death protein, and it has been shown that overexpression of murine ICE in rat fibroblast cells causes apoptosis, a process which can be prevented by co-expression of an inhibitor of ICE<sup>53</sup>. These studies raise the interesting possibility that inhibition of ICE may prove an effective treatment for many more pathological conditions than was previously thought.

### 1.1.7 Inhibition of ICE

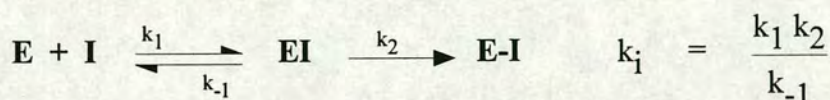
#### 1.1.7.1 Synthetic Inhibitors

All the synthetic inhibitors of ICE known to date are based on the tetrameric peptide minimum recognition sequence from proIL-1 $\beta$ ; no small organic molecules have yet been discovered that act as efficient inhibitors.

Reversible inhibition



Irreversible inhibition



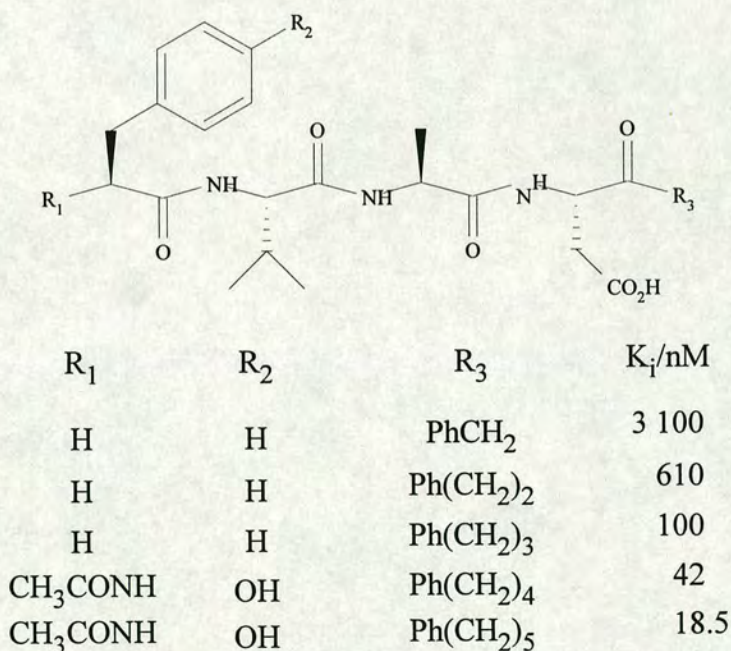
**Fig. 1.9. Reversible and irreversible enzyme inhibition**

Enzyme inhibitors fall into two classes, reversible and irreversible (Fig. 1.9). Peptide aldehydes are well known reversible inhibitors of serine and cysteine proteases; they mimic the tetrahedral intermediate formed during the enzyme-catalysed hydrolysis and are therefore known as transition-state analogues. The tetrapeptide aldehyde AcTyrValAlaAspH has been synthesised and found to be an inhibitor of ICE with a



$K_i$  of  $0.76 \pm 0.16 \text{ nM}^{54}$ . Whilst peptide aldehydes are often of low utility because of their low specificity, being inhibitors of both serine and cysteine proteases, the very unusual recognition requirements of ICE may make this a useful inhibitor.

A second class of reversible ICE inhibitors, the phenylalkyl ketones, have been developed by the same group<sup>55</sup>. The strategy was based on evidence for a hydrophobic binding pocket in the  $P_1'$ - $P_2'$  region; various ketones of the general type AcTyrValAlaAspR were prepared, where R was a phenylalkyl group and the length of the alkyl chain was varied. A dramatic increase in inhibitor potency was seen with increasing chain length (Fig. 1.10).

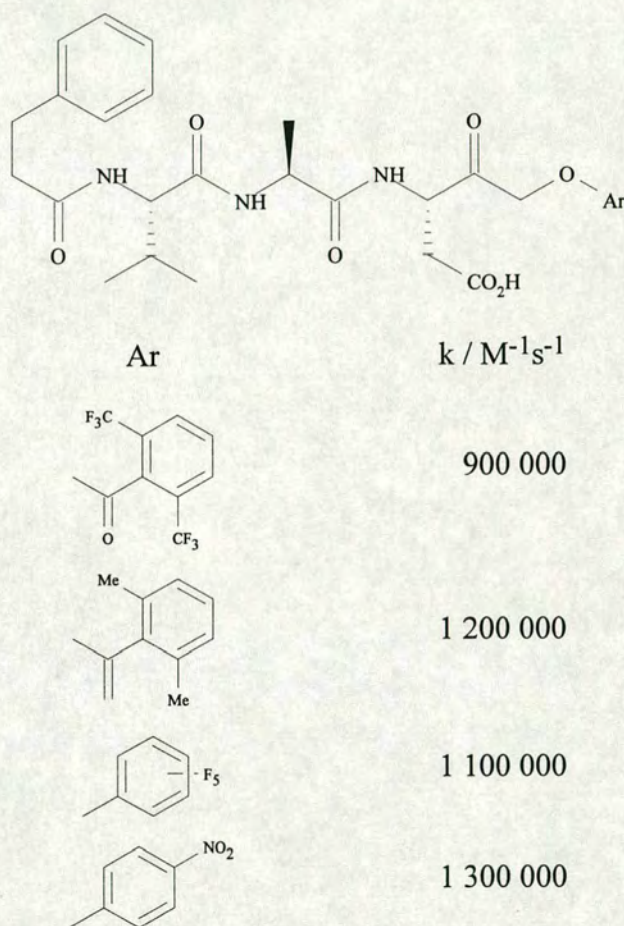


**Fig. 1.10. Phenylalkyl ketone ICE inhibitors**

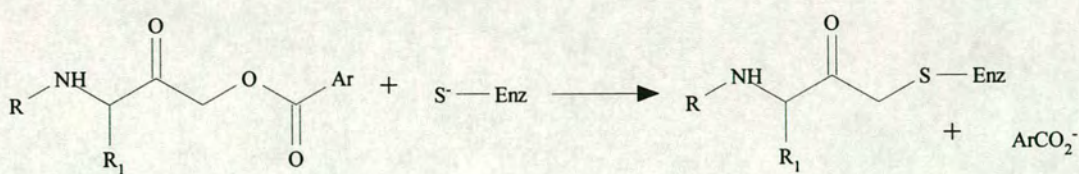
Irreversible enzyme inhibitors form a covalently bonded complex with the enzyme active site, their activity being described by the rate at which the enzyme is inactivated. The irreversible inhibitors of ICE described to date all take the form of a peptide methyl ketone, derivatised with a good leaving group: for example the fluoromethyl ketones, eg. AcHisAsp $\text{CH}_2\text{F}$  and AcTyrAsp $\text{CH}_2\text{F}^{56}$ , the diazomethyl



ketone AcTyrValAlaAspCH<sub>2</sub>N<sub>2</sub><sup>46</sup> ( $k=1.64\pm0.03\times10^4\text{M}^{-1}\text{s}^{-1}$ ) and the (acyloxy) methyl ketones<sup>57</sup> (Fig. 1.11). The detailed mechanism for inhibition by such species is not known; however, inactivation of cathepsin B via an appropriate (acyloxy) methyl ketone proceeds by expulsion of the aryl carboxylate leaving group to give a thiomethyl ketone (Fig. 1.12).



**Fig. 1.11. (Acyloxy) methyl ketone ICE inhibitors**



**Fig. 1.12. Inactivation of a cysteine protease by an (acyloxy) methyl ketone**



### 1.1.7.2 Natural Inhibition

It was noted previously (section 1.1.5.1) that the vaccinia and cowpox viruses encode a protein which inhibits the action of IL-1 by mimicking a soluble IL-1R. That such viruses adopt a multi-pronged approach to attacking the host defence system is demonstrated by the discovery that the cowpox virus also encodes a specific inhibitor of ICE<sup>58,59</sup> (Fig. 1.13).

The inhibitor is encoded by the cytokine response modifier gene, *crmA*. CrmA is a 38kDa protein which has been classed, from its amino acid sequence similarity, as a member of the serpin superfamily. The serpins are serine proteinase inhibitors; CrmA is the first cysteine protease inhibitor in the class.

Most serpins have a molecular weight in the range 45-100kDa; thus, at 38kDa, CrmA is smaller than other members of the superfamily. However, CrmA shares certain stability characteristics with the other inhibitory serpins. Serpins can exist in three conformational states, latent, cleaved and inhibitory, with only the latter state being active towards their target proteases. Serpins in their inhibitory state are very unstable, unfolding in 2-4M urea. CrmA undergoes such an unfolding transition in 3-4M urea<sup>86</sup>. However, in both its reactive site loop cleaved form and its complex with ICE it is stable to 8M urea, a situation also found for other serpins. Study of the kinetics of the ICE/CrmA interaction has found a  $K_i$  value of  $<4 \times 10^{-12}$ M and a half-life for complex dissociation of  $>160$  minutes at 37°C<sup>86</sup>.

Alignment of the sequence of CrmA with those of other serpins has led to the prediction of a reactive site. Interestingly, the tetrapeptide sequence corresponding to P4-P1 of the other serpins, LeuValAlaAsp, is very similar to the sequence contained in the optimum substrates, TyrValAlaAsp.



	MDIFREIASS	MKGENVFISP	PSISSVLITL	YYGANGSTAE
41	QLSKYVEKEA	DKNKDDISFK	SMNKVYGRYS	AVFKDSFLRK
91	TDCRTVDAIN	KCVDIFTEGK	INPLLDEPLS	PDTCLLAISA
141	FEKEFTSDYP	FYVSPTMVD	VSMMSMYGEA	FNHASVKESF
191	VGDTSMVVIL	PDNIDGLES	EQNLTDTNFK	KWCDSMDAMF
241	TGSYNLVDAL	VKLGLTEVFG	STGDYSNMCN	SDVSVDAMIH
291	TEAAAATCAL	VADCASTVTN	EFCADHPFIY	VIRHVDGKIL
				FVGRYCSPTT
	TEAAAATCALVADCASTVTNEFCAD			

**Fig. 1.13. The sequence and proposed reactive site of CrmA**

## 1.2 Solid Phase Peptide Synthesis

The synthesis of peptides is a challenge which has occupied chemists throughout the 20th Century, unsurprising given the role which peptides can play in advancing our understanding of many biological processes. Whilst recent years have seen the rise of biosynthetic methods of peptide and protein synthesis, the chemist will continue to have an important part to play; chemical synthesis allows the introduction of unnatural and D-amino acids, and of isotopic labels at specific residues, and continuing methodological advances now allow the rapid synthesis of milligram quantities of pure peptides of ever increasing molecular weight.

### 1.2.1 Strategies for Peptide Synthesis

The challenge of synthesising even small peptides by conventional chemical techniques is far from trivial, amino acids being by nature highly functionalised species. Taking as an example the synthesis of a dipeptide, AB, simply reacting the

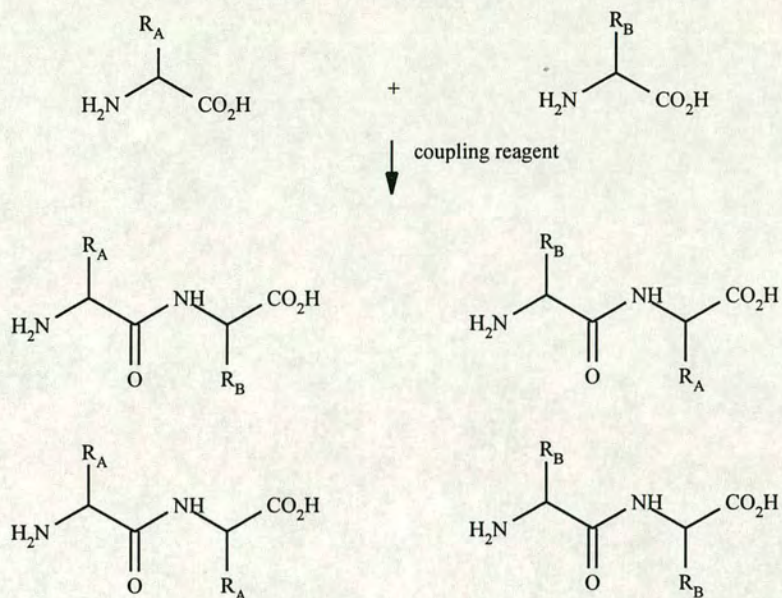


two amino acids in the presence of a coupling reagent will result in four possible dimers, only one of which is the correct product (Fig. 1.14), with the additional possibility of contamination by trimers, tetramers, etc. If the side-chains of amino acids A and B are also functionalised, as in, for example, lysine which carries an amino group, and aspartic or glutamic acid, carrying carboxylic acid functions, the possibility of cross-reactions increases accordingly. Clearly, then, protecting groups are required to ensure unambiguous synthesis, and, if the dimer AB is to be extended, the  $N^\alpha$ , side chain and carboxyl protecting groups must all be labile under different conditions, in addition to being stable to the conditions of the coupling reaction itself (Fig. 1.15).

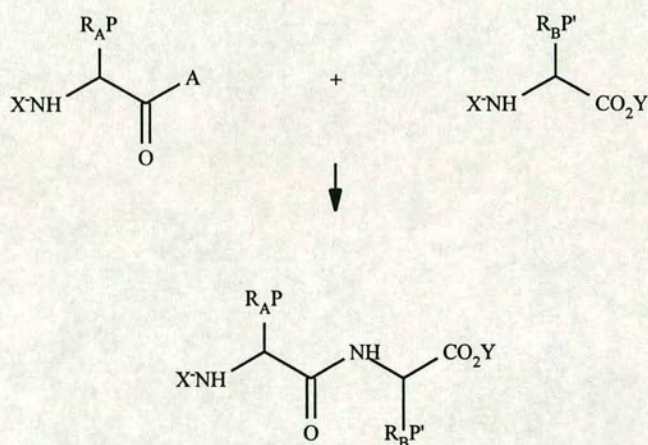
Larger peptides may be synthesised by one of three strategies: linear, either from N- to C-terminus (as in nature) or from C- to N-, or convergent (Fig. 1.16). Assuming that each of the steps shown in figure 1.16 gives a 90% yield, the overall yield of tetramer by the convergent route will be 73%, whereas for each of the linear routes it will be 66%. It would therefore appear that convergent synthesis by fragment condensation would be the method of choice. However, as will be discussed later, there are good chemical reasons for choosing to synthesise peptides linearly from the C-terminus (see section 1.2.4).

At each stage of a solution phase synthesis the product must be purified, by recrystallisation, chromatography, etc., a process which is both time-consuming and costly in terms of mechanical losses. Thus it is not hard to imagine that the synthesis of even a relatively small peptide might take a team of chemists several weeks. The potential for peptide synthesis was therefore revolutionised when, in 1959, Bruce Merrifield described the concept of solid phase peptide synthesis (SPPS).

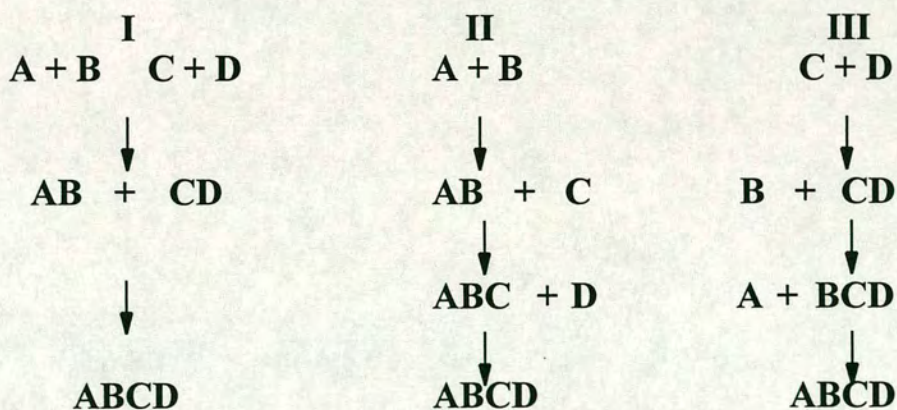




**Fig. 1.14. Undirected coupling of amino acids A and B**



**Fig. 1.15. Directed coupling of amino acids A and B**



**Fig. 1.16. Strategies for peptide synthesis**



In SPPS the C-terminal residue of a peptide chain is bonded covalently to an insoluble, polymeric support (referred to as 'resin'). The peptide is built up by a series of coupling steps in which an activated  $N^\alpha$ -protected amino acid is reacted with the free amino group at the N terminus of the growing peptide, alternated with deprotection steps to remove the  $N^\alpha$  protecting group. As the carboxyl terminal residue remains attached to the solid support throughout, the protecting group problem is reduced to the requirement for  $N^\alpha$  'temporary protecting groups' and 'permanent protecting groups' for the side-chains. Because the peptide is bonded to the insoluble support, purification at each stage becomes a question of simply washing away the unreacted reagents and by-products. This in turn means that each reaction can be driven to completion, or near completion, by the use of a large excess of reactants. The resin is retained in a single reaction vessel throughout the synthesis, thus minimising mechanical losses. Finally, the highly repetitive nature of the coupling and deprotection steps means that the process is readily amenable to automation. At the end of the synthesis the peptide is 'cleaved' from the resin and the permanent protecting groups removed, generally a concerted process.

The utility of SPPS was demonstrated by Merrifield in the early '60s by the synthesis of a tetrapeptide, Leu-Ala-Gly-Val<sup>60</sup>.

### 1.2.2 Protecting Group Strategies

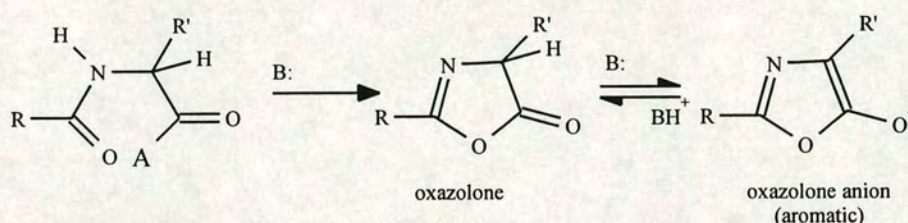
In Merrifield's first solid phase synthesis, no side-chain protection was necessary, since the four amino acids chosen all have aliphatic side chains. The  $N^\alpha$  protecting group used was the benzyloxycarbonyl (Z) group, which was removed by treatment with HBr in acetic acid, and the final cleavage from the resin was carried out with sodium hydroxide. Whilst this methodology was quickly changed to allow for less harsh conditions during the repetitive removal of the temporary protecting group, one



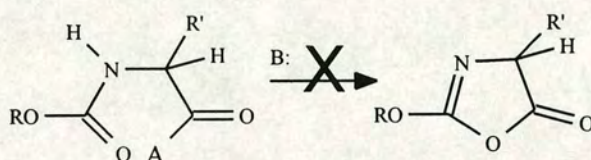
feature of the original Z group has been retained: all the major  $N^\alpha$  protecting groups are oxycarbonyls.

The protection of  $N^\alpha$  as a urethane prevents racemization of the activated amino acid through oxazolone formation, which can occur if the amino function is protected with an acyl group (Fig. 1.17). The oxazolone readily loses a proton to give an aromatic species in which stereochemical integrity is lost. Under normal conditions, urethanes will not form oxazolones, possibly due to the lower acidity of the urethane proton relative to that of the amide.

#### Acyl Protection



#### Oxycarbonyl Protection



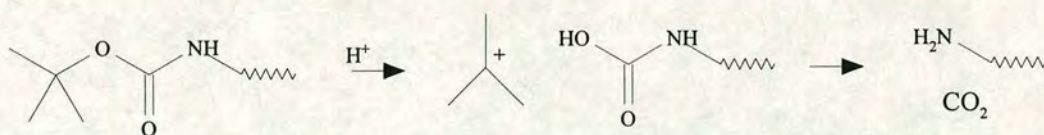
**Fig. 1.17. Amide vs. urethane protection**

### 1.2.2.1 Boc Methodology

The second  $N^\alpha$  protecting group used by Merrifield<sup>61</sup> was the *t*-butyloxycarbonyl (Boc) group<sup>62</sup>(3), which remains in widespread use today. The Boc group can be removed using a variety of acidic reagents, eg. 0.1N HCl/acetic acid, neat trifluoroacetic acid (TFA) or TFA/DCM in various ratios from 1:4 to 1:1. Initial loss



of the *t*-butyl cation is followed by spontaneous decomposition of the carbamic acid to free amine and CO<sub>2</sub> (Fig. 1.18).



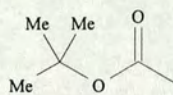
**Fig. 1.18. Deprotection of a Boc-protected peptide**

The greater stability of the *t*-butyl cation in comparison with the benzyl cation means that the Boc group can be removed under milder acidic conditions than the Z group. This in turn means that, when Boc is used for N<sup>α</sup> protection, protecting groups based on the Z group, ie. benzyl esters, ethers and Z itself, can be used for side-chain protection, since these will be stable to the acid conditions used for N<sup>α</sup> deprotection throughout the synthesis. Final side-chain deprotection is carried out using very strong acid (usually liquid HF).

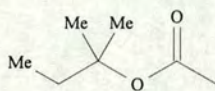
Numerous N<sup>α</sup> protecting groups based on Boc have been developed, examples being *t*-amyloxycarbonyl (Aoc)<sup>63</sup>(4), adamantyloxycarbonyl (Adoc)<sup>64</sup>(5), 2-(*p*-biphenyl)propyl-(2)-oxycarbonyl (Bpoc)<sup>65</sup>(6) and α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl (Ddz)<sup>66</sup>(7).

In general, Aoc and Adoc have very similar properties to Boc. However, their derivatives have more favourable solubility characteristics and are more easily prepared due to the greater stability of the chloroformates, and therefore may be preferred under certain circumstances. On the other hand, Ddz and Bpoc were designed to be far more acid labile than Boc. The relative deprotection rates in 0.5% TFA/DCM are<sup>67</sup> Boc:Ddz:Bpoc 1:2.3x10<sup>4</sup>:1.3x10<sup>5</sup>. Therefore Bpoc and Ddz can be used in conjunction with *t*-butyl-derived side-chain protecting groups, allowing for a far milder synthesis overall.

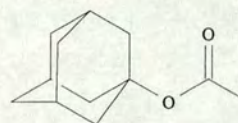




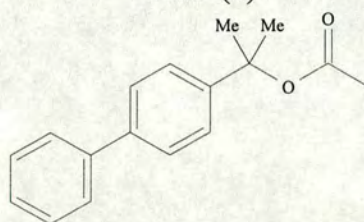
Boc (3)



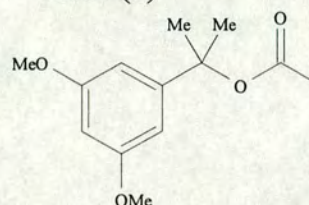
Aoc (4)



Adoc (5)



Bpoc (6)



Ddz (7)

### 1.2.2.2 Fmoc Methodology

The 9-fluorenylmethyloxycarbonyl (Fmoc)<sup>68</sup>  $N^\alpha$  protecting group allows a truly orthogonal approach to SPPS as it is stable to acid and catalytic hydrogenation, but is removed on treatment with mild base (20% piperidine/DMF) by a  $\beta$ -elimination mechanism (Fig. 1.19). Thus Fmoc temporary protection can be combined with *t*-butyl-derived side-chain protection.

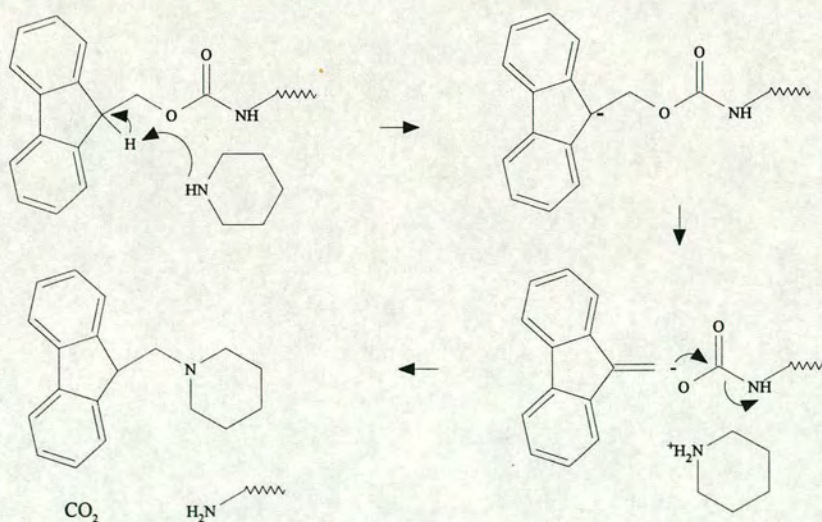


Fig. 1.19. Deprotection of an Fmoc-protected peptide



The dibenzofulvene deprotection intermediate is trapped by excess piperidine, preventing it from reacting with free amine, and giving an adduct which has a strong absorbance at 300nm. This forms the basis of a system for monitoring the success of the synthesis, as the extent of coupling of each residue can be calculated from the UV absorbance of the filtrate after each deprotection<sup>69</sup>.

	Boc Methodology	Fmoc Methodology
-CO <sub>2</sub> H	-CO <sub>2</sub> CH <sub>2</sub> Ph	-CO <sub>2</sub> <sup>t</sup> Bu
-OH	-OCH <sub>2</sub> Ph	-O <sup>t</sup> Bu
-NH <sub>2</sub>	-NHCO <sub>2</sub> CH <sub>2</sub> Ph	-NHCO <sub>2</sub> <sup>t</sup> Bu

**Table 1.2. Standard side-chain protecting group strategies for SPPS**

### 1.2.3 The Solid Support

The most successful solid supports developed to date are the polyamides<sup>70</sup> and copolymers of styrene with divinylbenzene (0.5-2%). Polystyrene resins are functionalised by chloromethylation. For a successful synthesis it is necessary that the resin swells effectively in the solvents used since the overwhelming majority (>99.9%) of the functional sites are located on the interior of the polymeric matrix<sup>71</sup>.

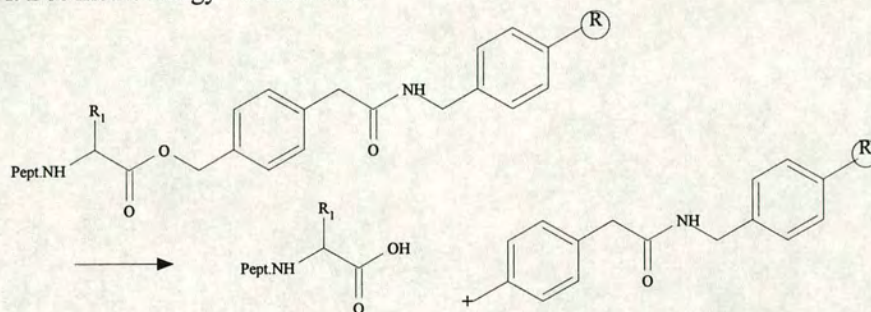
In the first solid phase synthesis the peptide was attached directly to the functionalised resin. However, the introduction of 'linkers' between the support and the peptide has allowed fine-tuning of the strength of the peptide-resin bond, so that it can be made more or less labile to suit the synthetic strategy. This is demonstrated by the linkers used in Boc and Fmoc methodologies (Fig. 1.20). In Boc methodology the peptide-linker bond is a benzyl ester, allowing it to be cleaved simultaneously with the side chain protecting groups during HF treatment. However, the Wang linker<sup>72</sup> generally used in Fmoc methodology is cleaved by TFA along with the <sup>t</sup>Bu-



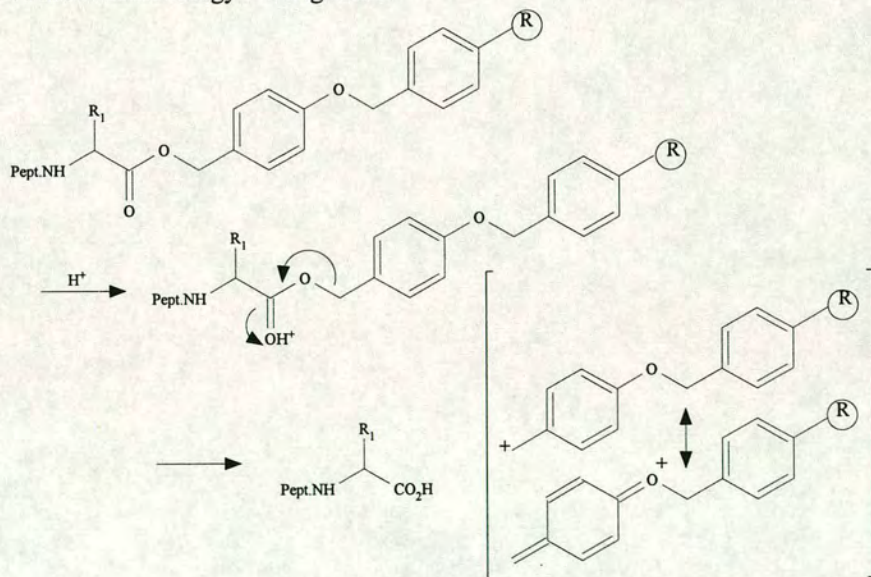
derived protecting groups. In both cases the cleavage mechanism is an ester hydrolysis by alkyl-oxygen fission, rather than the more common acyl-oxygen fission. In the *p*-benzyloxybenzylalcohol (Wang) resin the additional *p*-oxygen helps to stabilise the carbocation formed during the fission process, thus increasing the lability of the peptide.

The use of linkers also allows the functionality at the C-terminus of the cleaved peptide to be adapted to requirements. Resins are available that give peptide amides and peptide hydrazides under standard cleavage conditions.

A. Boc methodology - PAM Resin



B. Fmoc Methodology - Wang Resin

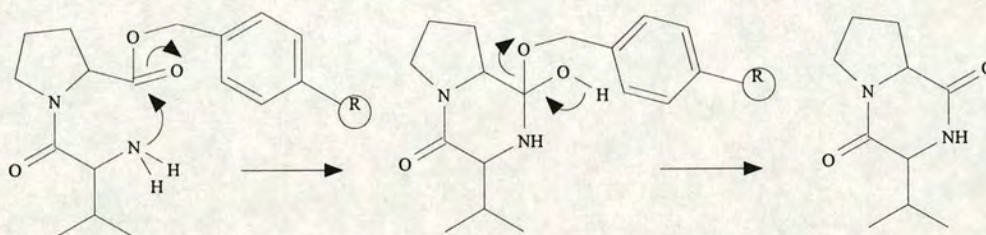


**Fig. 1.20. Acidolytic cleavage from the resin**



### 1.2.3.1 Diketopiperazine Formation

The benzyl alcohol moiety of the peptide-resin ester linkage is, under normal conditions, a poor leaving group in  $S_N2$  reactions. The only significant nucleophiles present during a synthesis are the free amino terminal groups of the growing peptide chain, and therefore during most of the synthesis the peptide-resin anchor is almost 100% resistant to attack. However, at the level of the dipeptide, the amino terminus is uniquely positioned to undergo an intramolecular cyclization to form a 6-membered diketopiperazine ring<sup>73</sup> (Fig. 1.21), effectively terminating the peptide chain.



**Fig. 1.21. Diketopiperazine formation in the dipeptide Val-Pro-Resin**

Diketopiperazine formation is strongly sequence-dependent, as the transition state requires that the peptide bond adopt the, usually disfavoured, *cis*-conformation. For a given C-terminal residue the likelihood of the rearrangement occurring is in the order N-methyl amino acid>Pro>Gly>Val. In general the side reaction can be ignored as it occurs at a level of <5%, unless either of the first two residues is glycine or proline.

### 1.2.4 Coupling

The coupling of two amino acids always occurs by attack of the nucleophilic  $\alpha$ -amino function on an activated carboxyl function; there is no practical coupling method available that proceeds through an activated amino function. The degree of

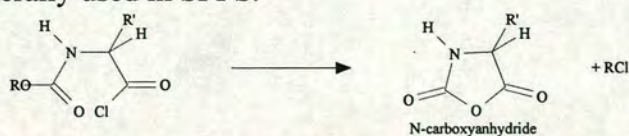


activation controls the rate of coupling, but also the likelihood of side reactions, so that a delicate balance between the two must be maintained.

At this point it is worth mentioning the reasoning behind the synthesis of peptides from the C- to N-terminus, rather than nature's way (N to C). Since it is always the carboxyl group which is activated, in an N to C synthesis it is the growing peptide chain which will be activated. However, the  $N^\alpha$  group of the C-terminal residue in a peptide is effectively 'protected' by an acyl group, making it susceptible to racemization through the oxazolone (see Fig. 1.17). Thus some racemization can be expected at every coupling step, and the racemized product is attached to the solid support, resulting in a purification nightmare for the final cleaved product. In a C to N synthesis, however, the activated species is the solution-phase amino acid. As has already been described, use of urethane-type protection in such a species effectively prevents racemization. In addition, should the activated amino acid undergo any rearrangement that prevents it from reacting, it will simply be washed away at the end of the coupling step, not, therefore, interfering in the synthesis.

#### 1.2.4.1 Acid Chlorides

The most obvious method of forming an amide from a carboxylic acid and an amine is through the acid chloride. However, a urethane-protected amino acid chloride is an inherently unstable species, having a tendency to form N-carboxyanhydrides<sup>74</sup> (Fig. 1.22). In addition, the reaction conditions required for acid chloride formation and coupling are incompatible with  $t$ Bu-based protecting groups, and therefore this method is not generally used in SPPS.

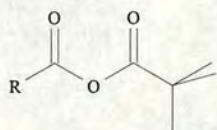


**Fig. 1.22. Decomposition of a urethane-protected amino acid chloride**

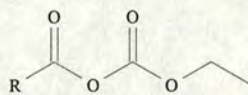


#### 1.2.4.2 Mixed Anhydrides

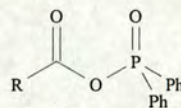
Three types of mixed anhydrides have been used in peptide synthesis, those of carboxylic acids, carbonic acids and phosphorous derivatives.



(8)



(9)



(10)

Mixed anhydrides of carboxylic acids have two electrophilic sites which can lead to ambiguous nucleophilic attack. For this reason the main mixed anhydrides of this type used are the mixed pivalic anhydrides (8) in which both steric hinderance and the electron-donating inductive effect of the *t*-butyl group direct attack to the required carbonyl. In the carbonic acid mixed anhydrides (9) the carbonyl group that is not required to react is flanked by two oxygens, thus decreasing its reactivity and increasing the regioselectivity of the nucleophilic attack. However, both these classes of mixed anhydride have a major disadvantage as far as SPPS is concerned: they must be used at low temperatures (-20°C to -5°C) as they have a tendency to disproportionate, and commercial peptide synthesisers are designed to operate at room temperature. The mixed carboxylic-phosphinic anhydride (10) circumvents both these problems, being far more stable at ambient temperatures and undergoing nucleophilic attack exclusively at the carbonyl.

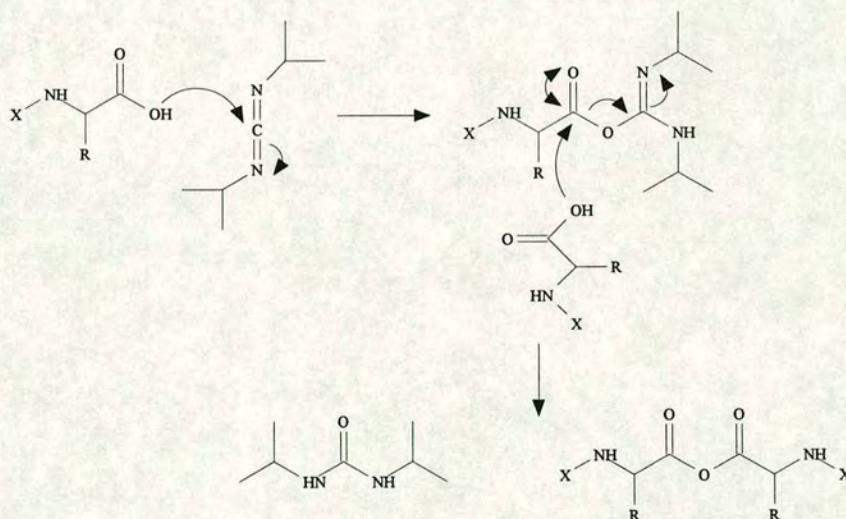
#### 1.2.4.3 Symmetrical Anhydrides

Symmetrical anhydrides have the advantage over mixed anhydrides that there is no possibility of chain termination due to opening of the anhydride the 'wrong way'. It is an expensive method, as half the protected amino acid species is unused, but is nonetheless useful in SPPS as the highly activated species couples very rapidly<sup>75</sup>.

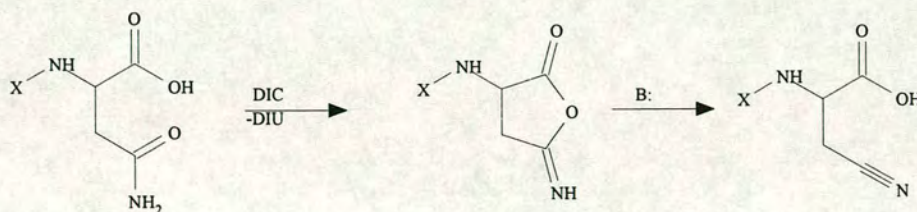


Symmetrical anhydrides are generally preformed immediately before use, by the action of a carbodiimide on two equivalents of the protected amino acid. Originally dicyclohexylcarbodiimide (DCC) was used for this purpose, but the current reagent of choice is diisopropylcarbodiimide (DIC) since the by-product of the reaction, diisopropylurea, is fully soluble in the solvents used in SPPS (Fig. 1.23).

Side-chain-unprotected asparagine and glutamine cannot be coupled by this method, as their amide groups are susceptible to dehydration to a nitrile functionality by both DCC and DIC (Fig. 1.24).



**Fig. 1.23. Formation of a symmetrical anhydride by the action of DIC**

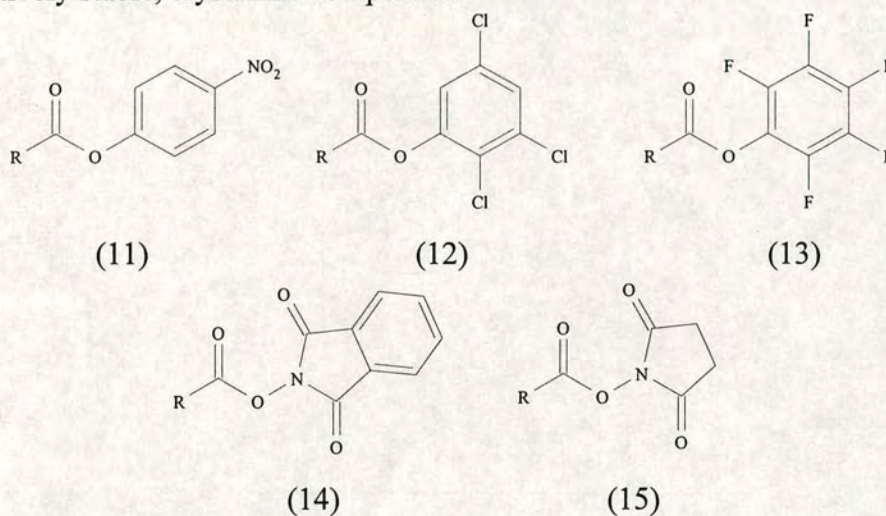


**Fig. 1.24. Dehydration of asparagine by the action of DIC**

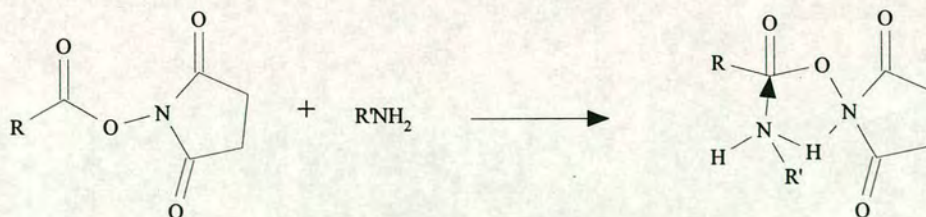


#### 1.2.4.4 Activated Esters

The coupling rates of activated esters are considerably lower than those of the symmetrical anhydrides, but they have the advantage that none of the amino acid derivative is 'wasted'. Activated esters have been used extensively in solution-phase peptide chemistry, the phenyl esters in particular, eg. *p*-nitrophenyl<sup>76</sup>(11), 2,4,5-trichlorophenyl<sup>77</sup>(12) and pentafluorophenyl<sup>78</sup>(13), having the the advantage that they are relatively stable, crystalline compounds.



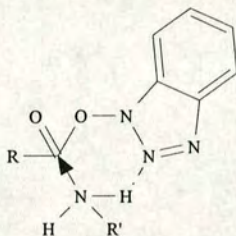
A second class of activated esters important in solution-phase chemistry comprises the derivatives of N-hydroxyphthalimide<sup>79</sup>(14) and N-hydroxysuccinimide<sup>80</sup>(15). Such N-acylhydroxylamines (or hydroxamic acids) are acidic compounds and their O-acyl derivatives could be thought of as mixed anhydrides. However, their reactivity is due rather to interactions between the incoming amine and the nitrogen atom of the leaving group (Fig. 1.25).



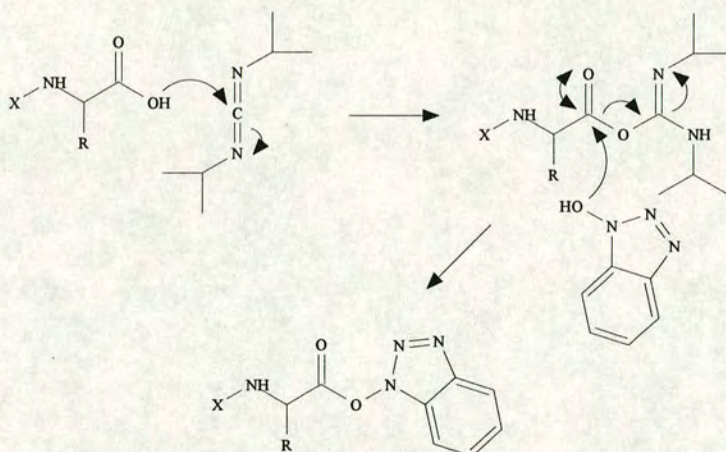
**Fig. 1.25. Interaction of an N-hydroxysuccinimide AE with an incoming amine**



Despite the utility of the above reagents in solution-phase peptide synthesis, they were found to be ineffective in SPPS. Fortunately, however, it was discovered that 1-hydroxybenzotriazole (HOBt)<sup>81</sup>, originally added to coupling reactions to prevent racemization, forms an activated ester which can be used successfully in SPPS. The HOBt derivative interacts with the incoming amine in an analogous fashion to the hydroxamic acid derivatives (Fig. 1.26). HOBt activated esters are preformed immediately before use in the presence of one equivalent of DIC (Fig. 1.27).

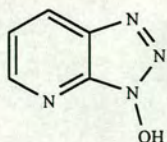


**Fig. 1.26. Interaction of an HOBt AE with an incoming amide**

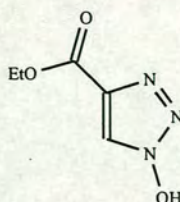


**Fig. 1.27. Formation of an HOBt activated ester by the action of DIC**

The success of HOBt in SPPS has led to the development of improved analogues, 1-hydroxy-7-azabenzotriazole (HOAt)<sup>82</sup> (16) and ethyl-1-hydroxy-1H-1,2,3-triazole-4-carboxylate (HOCT)<sup>83</sup> (17).



(16)



(17)



## 2. Discussion

### 2.1. Introduction

In the search for a therapeutic strategy for the combat of rheumatoid arthritis, IL-1 $\beta$  appears to be a good target since it has been shown to effect many of the pathologies of the disease. Intervention could concentrate on blocking the binding of IL-1 $\beta$  to its receptor; trials involving the use of IL-1ra to such an end have been carried out<sup>35</sup>. However, due to the low receptor occupancy required for IL-1 $\beta$  to exert its effect, very large excesses of a receptor antagonist would be required for successful treatment by this method. Since IL-1 $\beta$  is biologically inactive in its precursor state, inhibiting the IL-1 $\beta$  converting enzyme (ICE) should effectively prevent those symptoms arising from the action of extracellular IL-1.

Considerable effort has been expended in the search for a small, non-peptidic inhibitor of ICE. To date none has been reported. Therefore the aim of this work has been to attempt to establish factors of importance for substrate recognition by ICE, beyond those found in previous work<sup>43,44,45</sup> (see section 1.1.6), by the use of solid phase peptide synthesis.

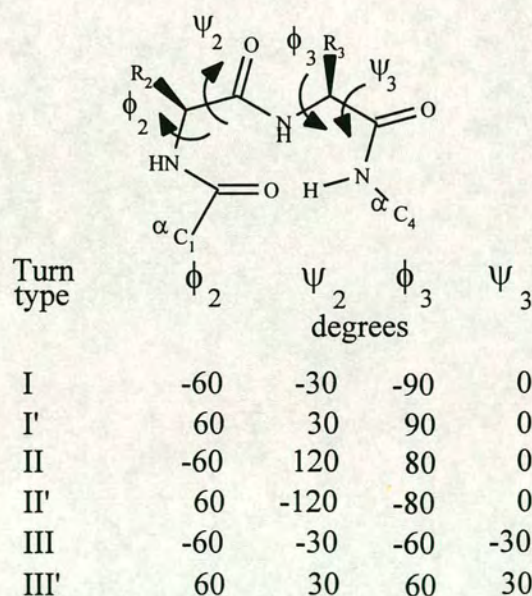
The work has been concentrated in three areas, the cleavage region of proIL-1 $\beta$ , the cowpox inhibitor crmA, both of which are known to interact with ICE, and the pro region of ICE itself, which may be important for correct folding of the enzyme.



## 2.2. ProIL-1 $\beta$ Peptides

Inspection of the crystal structure of mature IL-1 $\beta$  reveals that the residues immediately following the cleavage site form a  $\beta$ -sheet with residues from  $\beta$ -strand 4 (see Fig 1.2). It was decided to attempt the synthesis of a peptide incorporating both the cleavage site and the relevant strand 4 residues (160-163), the fragments being linked by a  $\beta$ -turn-like segment.

The  $\beta$ -turn is a common structural motif in proteins, being a tetrapeptide sequence which changes the direction of the peptide chain by 180°. Several classes of  $\beta$ -turn have been described, based on the torsion angles of the backbone residues 2 and 3<sup>87</sup> (Fig 2.1). A hydrogen bond between the carbonyl oxygen of residue 1 and the NH of residue 4 stabilises the conformation.



**Fig 2.1. The structure of the  $\beta$ -turn and the classification of the major types**



In the first instance it was decided that the 180° turn required in the test peptide could be fixed by the formation of a disulphide bond between two cysteine residues. In order to facilitate the formation of the S-S bond the Cys residues were to be separated by two amino acids, two glycine residues being chosen.

The synthesis of KU1, proIL-1 $\beta$  113-122-CysGlyGlyCys-160-163 (YVHDAPVRSL CGGCMSFV), was carried out using standard methodology, the cysteines being protected with the acid-labile trityl group, which is removed concurrently with cleavage from the resin. The synthesis was disappointing, with a large drop of >40% in the coupling efficiency of <sup>10</sup>Leu, and a further decrease over the next three residues, so that the final Fmoc deprotection showed overall coupling of just 36%.

Despite the poor synthesis an initial, small-scale cleavage gave some encouragement, resulting in two easily separable products, the required peptide and the expected main deletion product, CGGCMSFV. However, scale-up gave an altogether more complex crude product. It was assumed that this was due to dimerisation of the peptide, despite the fact that the work-up was carried out using solvents containing 2-5% dithiothreitol (DTT) in an attempt to keep the free thiols in their reduced state.

Oxidation to the cystine-containing peptide also proved difficult. Simple air oxidation at low peptide concentration resulted in no discernible change in the analytical HPLC. A DMSO oxidation<sup>88</sup> was attempted, though this was risky given the presence of a highly oxidation-sensitive methionine residue. Perhaps



unsurprisingly, treatment of the peptide with DMSO resulted in an unworkably complex mixture of products, as shown by analytical HPLC. In view of the problems with the synthesis, cleavage and oxidation, this synthesis was abandoned.

On repeating the synthesis it was decided to employ cysteine protecting groups which could be removed independently of the other side-chain protection, and hence the *tert*-butylsulphenyl (S<sup>t</sup>Bu) group was chosen. In addition the two glycine residues were replaced by proline-D-valine, a dipeptide unit known to favour the formation of type II'  $\beta$ -turns<sup>89</sup>, as it was thought that this might facilitate the oxidation step. The synthesis of KU2 was carried out as before, except that six residues, <sup>8</sup>Arg-<sup>13</sup>D.Val, were given extended coupling times in order to try to avoid the drop in coupling efficiency seen previously, and because the behaviour of the peptide containing the  $\beta$ -turn-inducing residues was unpredictable. This strategy was successful, the coupling efficiency of the penultimate residue being 99%.

It has been reported that cyclisation of a dicysteine-containing peptide can be accomplished whilst the peptide remains anchored to the solid support<sup>90</sup>. This methodology takes advantage of the phenomenon of pseudo-dilution: whilst a peptide chain is attached to the resin it is effectively unable to interact with other peptides and hence can only react intramolecularly. The removal of S<sup>t</sup>Bu groups from the resin-bound peptide was carried out by treating preswollen resin with tributylphosphine for 5-23 hours. Cleavage of treated resin showed that up to 50% of the peptide was fully deprotected; however, it was extremely difficult to obtain



reproducible results. The oxidation method described by Eritja *et al*<sup>90</sup> involved the treatment of DMF-swollen resin with an aqueous solution of potassium ferricyanide. However, most of the potassium ferricyanide appeared to precipitate on contact with the DMF, and perhaps unsurprisingly no oxidation was seen. In view of the difficulties experienced, the method was not pursued.

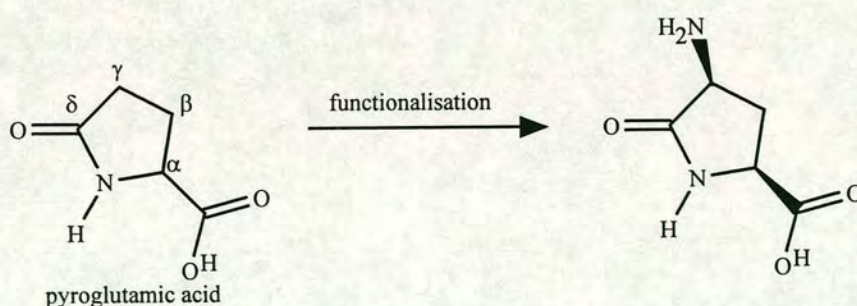
Fortunately the procurement of the required peptide by conventional methods proved far easier. Cysteine-protected KU2 was readily purified by preparative HPLC, and subsequent removal of the S<sup>t</sup>Bu groups was completed in 2.5 hours by treatment with tributylphosphine. The oxidation was carried out cleanly by an air oxidation in aqueous buffer at pH8-9. A low concentration, 1mg in 10ml, was used to prevent dimerisation<sup>91</sup>.

Whilst the synthesis of KU2 was successful, the formation of the disulphide bond was both time-consuming and costly in terms of the mechanical losses associated with the sulphur deprotection and oxidation steps. Clearly, therefore, it would be advantageous if a single entity could be found which could be introduced into the peptide chain during synthesis, and which would promote the required 180° change of direction, namely a  $\beta$ -turn mimic.

Unlike the major types of  $\beta$ -turn (I,II & III and their inverses) in which all the peptide bonds are of the usual *trans* type, the typeVI turn contains a sterically less favourable *cis* peptide bond between residues 2 and 3. Paul *et al*<sup>92</sup> have suggested

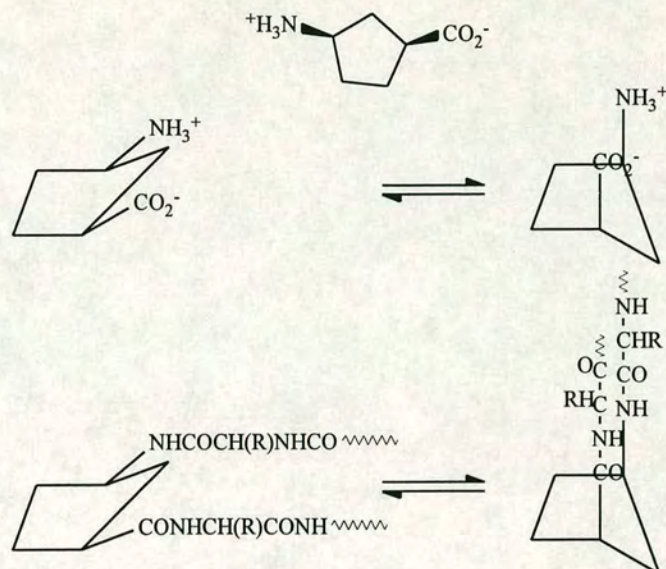


that a pyroglutamic acid residue functionalised at C<sup>γ</sup> with an amino group *cis* to the carboxylate (Fig 2.2) may act as a β-turn mimic, since modelling studies indicate that such a molecule adopts a conformation with  $\phi$  and  $\psi$  values typical of a type VI turn. On the basis of these observations it is proposed that a simple cyclopentane ring system, bisubstituted with an amino and a carboxylic acid group may also act as a type VI β-turn mimic. It is expected that the *pseudo*-diequatorial form of *cis*-aminocyclopentane-1-carboxylic acid (ACPC) would be energetically favoured compared with the *pseudo*-diaxial form; however, on incorporation of ACPC into a peptide, hydrogen-bonding interactions between the two strands of a β-sheet structure should increase the stability of the diaxial conformation (Fig. 2.3). In addition, it is suggested that the flexibility of the system may give it an advantage, in certain situations, over other, more rigid β-turn mimics which have been described in the literature<sup>93</sup>. The nature of interactions of peptides and proteins with enzymes and receptors is such that the conformation of the protein in solution may be different from its conformation in the bound state. Therefore, a degree of flexibility in the β-turn mimic may allow the peptide to adopt the required conformation more readily.



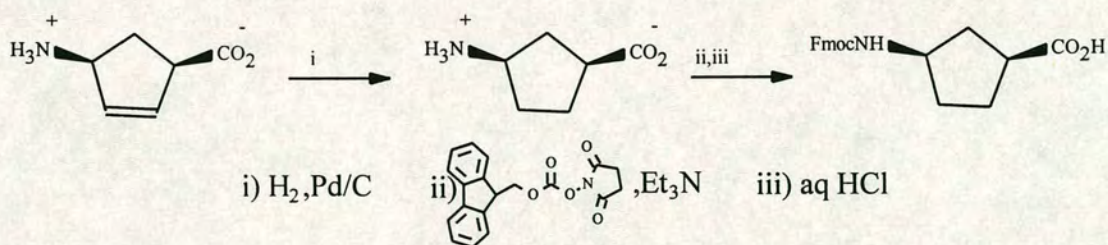
**Fig. 2.2. Functionalisation of a pyroglutamic acid residue to produce a potential type VI β-turn mimic.**





**Fig 2.3. *cis*-3-aminocyclopentane-1-carboxylic acid (ACPC) - a proposed type VI  $\beta$ -turn mimic.**

As the  $\beta$ -turn mimic, ACPC, is a  $\beta$ -amino acid it is amenable to insertion into peptides via standard Fmoc methodology. The Fmoc derivative was obtained in two steps, by the hydrogenation of *cis*-3-aminocyclopent-4-ene-1-carboxylic acid, followed by Fmoc protection of the amino group by treatment with 9-fluorenylmethyl succinimidyl carbonate in the presence of base (Fig. 2.4).



**Fig. 2.4. Synthesis of FmocACPC**

The synthesis of KU3, proIL-1 $\beta$ 113-122-ACPC-160-163, was the first to employ FmocACPC. Therefore the first few residues, up to and including the leucine residue coupled to the ACPC-peptide-resin, were coupled manually using bubbler apparatus.



This allowed the coupling of each residue to be checked by a Kaiser test before Fmoc deprotection to ensure a quantitative synthesis. Coupling of FmocACPC was carried out using the HOCT activated ester. This coupling reagent was developed in this laboratory, and has been shown to give faster, more efficient couplings than HOBt<sup>83</sup>. The Kaiser test revealed 100% coupling after a single, overnight treatment. The synthesis was completed automatically and analytical HPLC of the crude product showed a very efficient synthesis. Simple preparative HPLC gave the required peptide.

In order to be able to establish the effect of the imposed  $\beta$ -sheet structure on the recognition of KU2 and KU3 by ICE, it was decided that the peptide comprising only the cleavage region of proIL-1 $\beta$  should be synthesised. Therefore the 8-mer, proIL-1 $\beta$  113-120 (KU4), which has been shown to be the optimum small peptide substrate of ICE<sup>43</sup>, was synthesised using standard techniques.

Whilst it is known that a cleavage site peptide substituted with a glutamic acid residue at the P<sub>1</sub> (Asp) site is a poor substrate for ICE (see Fig. 1.5), no K<sub>i</sub> value showing the ability of such a peptide to act as an inhibitor of ICE has been published. Therefore the peptide proIL-1 $\beta$  111-122 <sup>116</sup>Glu was synthesised for comparison of its inhibitory efficacy with that of proIL-1 $\beta$  111-122 itself, as synthesised in this laboratory by D Maclean<sup>45</sup>.



Whereas the crucial residue for recognition of the proIL-1 $\beta$  processing site by ICE is aspartic acid, the majority of known prohormones and propeptides are cleaved C-terminal to a pair of basic residues (arginine and/or lysine). However, just as ICE does not cleave at all Asp residues, enzymes such as trypsin do not recognise all dibasic sites. There is evidence that propeptides are cleaved at dibasic sites that are immediately preceded by  $\beta$ -turns<sup>94</sup>, although whether the type of turn is important has not been elucidated. This raises the possibility that some secondary structural element is also important for processing site recognition by ICE. Thus it is interesting that both the sites at which proIL-1 $\beta$  is cleaved, Asp27-Gly28 and Asp116-Ala117, are immediately succeeded by proline, a residue known to encourage the formation of  $\beta$ -turns. Changing the configuration of an amino acid residue will inevitably change the conformation of a  $\beta$ -turn in which it is involved. Therefore the peptide proIL-1 $\beta$  111-122 <sup>119</sup>D.Val (KU6) was synthesised in an attempt to discover whether the secondary structure in this region is of importance.

Y V H D A P V R S L C PdV C M S F V	KU2
Y V H D A P V R S L (ACPC) M S F V	KU3
Y V H D A P V R	KU4
E A Y V H E A P V R S L	KU5
E A Y V H D A PdV R S L	KU6

**Table 2.1. ProIL-1 $\beta$  Peptides**

### 2.3. CrmA Peptides

The reactive site loop (RSL), through which serpins bind to and inhibit proteases, is a flexible region in an otherwise structurally well-defined molecule<sup>95</sup>. Thus it seems



likely that the conformation required for inhibition is induced on complexation of the serpin with the enzyme. Therefore it was of interest to discover whether a short peptide representing the RSL of CrmA, and almost certainly being inherently unstructured, would be recognised by ICE, and if so whether it would act as a substrate, or as a true inhibitor.

Sleath *et al*<sup>43</sup> found the peptide comprising the four residues on each side of the proIL-1 $\beta$  cleavage site (P<sub>4</sub>-P<sub>4</sub>') to be preferred as a substrate of ICE compared with both longer and shorter sequences. Therefore in the first instance the 8-mer corresponding to these residues in the RSL, CrmA 300-307 (KU7), was synthesised. In this sequence the P<sub>1</sub> Asp is followed by a cysteine residue. The preferred P<sub>1</sub>' residue in ICE substrates is Ala or Gly. It seems possible, therefore, that the cysteine is involved in the inhibitory process in some way. To test this theory a second 8-mer (KU8) was synthesised, in which the order of the P<sub>1</sub>' and P<sub>2</sub>' residues, CysAla, was reversed, creating a classic ICE recognition site. It was hoped that, if inhibition were seen with CrmA 300-307, this change would result in restoration of substrate activity.

Finally a slightly longer peptide, CrmA 298-309 (KU9), was synthesised. There were several reasons for this. Firstly, it was hoped to see whether variation in length affects recognition in an analogous fashion to that seen for fragments of proIL-1 $\beta$ . Secondly, the longer peptide contains a second Cys residue at the P<sub>6</sub> position. It was hoped that it would be possible to oxidise the peptide to form a disulphide bond



between this and the P<sub>1</sub>' cysteine, thus imposing structure on it. If the imposed structure resembled that of the inhibitory conformation of the RSL then improved inhibition might be seen. Additionally, since the structure of CrmA is unknown, it is not certain whether the Cys residue at the P<sub>1</sub>' position exists as a free cysteine, or is involved in a disulphide bond. Therefore it was thought possible that the oxidised peptide might have different inhibitory/substrate properties compared with the reduced form. Finally, cyclic peptides are often more stable than their linear analogues, having enhanced resistance to proteolytic degradation<sup>96</sup>.

AcLVADCAST-NH <sub>2</sub>	<b>KU7</b>
AcLVADACST-NH <sub>2</sub>	<b>KU8</b>
AcCALVADCASTVT-NH <sub>2</sub>	<b>KU9</b>

**Table 2.2. CrmA Peptides**

The synthesis of KU7 was rather disappointing, the estimated final coupling efficiency of about 50% being poor for such a small peptide. Clearly the sequence is a difficult one. Therefore for the synthesis of KU8 the final five residues were given extended coupling times, and the most problematic residues, <sup>1</sup>Leu and <sup>5</sup>Ala, were triple coupled. The synthesis was interrupted before removal of the final Fmoc so that the extent of coupling could be checked manually. This revealed that the coupling efficiency for the leucine was just 57%. This residue was therefore recoupled as the HOCT activated ester, a strategy which had been found to improve the synthesis of other difficult sequences<sup>97</sup>. However, in this case no improvement



was seen in the coupling efficiency. Finally, the resin was sonicated with 4 equivalents of the Fmoc-leucine HOBt activated ester, overnight. This brought the final coupling efficiency to a more acceptable >90%.

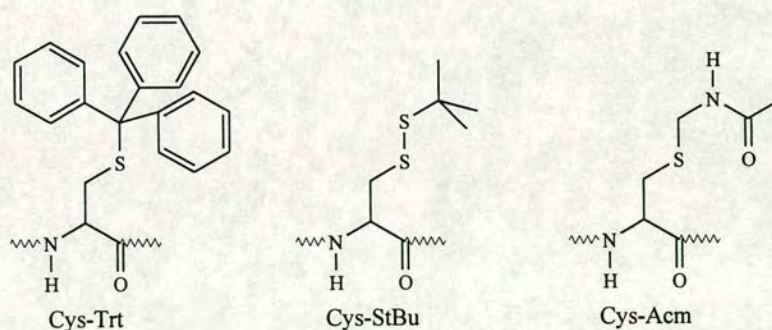
The longer CrmA fragment was initially synthesised as the 13-mer, CrmA 297-309, AcThrCysAlaLeuValAlaAspCysAlaSerThrVal ThrNH<sub>2</sub> (KU10). The 12-mer, CrmA 298-309, has an N-terminal cysteine residue. These can sometimes be problematic, leading to facile dimerisation of the peptide. However, the synthesis of KU10 was poor, with a significant drop (30%) in coupling efficiency over the internal residues <sup>7</sup>Asp-<sup>5</sup>Val, and a disastrously poor coupling of the final threonine residue. In particular, it would have been difficult to separate the required peptide from the *des*-<sup>1</sup>Thr deletion peptide by preparative HPLC. Therefore the synthesis was repeated, omitting the final Thr residue, and extending the coupling times of the problematic, central residues.

Problems were also encountered during the purification of these peptides. Since KU7 and KU8 contained free thiol groups, dithiothreitol (2-5%) was added to all solvents used in the cleavage work-up and when dissolving samples for HPLC, in order to prevent dimerisation. The crude peptides were very insoluble which made preparative HPLC difficult. Prior to injection onto a semi-preparative HPLC column, a peptide must be dissolved at a concentration of 5-10mg/ml. Various combinations of water, acetic acid and acetonitrile were tried, without success. Eventually it was discovered that the peptides could be dissolved satisfactorily by



suspending them in aqueous 6M guanidine hydrochloride and adjusting the pH to between 8.5 and 9 by the addition of solid Trizma Base (Sigma). For KU7, preparative HPLC was carried out using both the standard, acidic buffer system and a basic buffer system comprising Buffer A: 50mM aqueous ammonium acetate, pH8.5; Buffer B: 10% A/acetonitrile. As the standard TFA buffered system gave more satisfactory results, this was used throughout the purification of the remaining peptides.

The two 8-mers contained only one cysteine residue apiece, and the trityl (Trt) protecting group, which is removed on TFA cleavage, was used for these. However, as the 12-mer, KU9, contains two cysteine residues, and in particular an N-terminal one, it was felt that it would be advisable to be able to control the removal of the cysteine protection. Since the sequence in question proved to have such low solubility, the synthesis of KU9 was carried out using the solubilising acetamidomethyl (Acm) protecting groups, rather than the very hydrophobic *tert*-butylsulphenyl (S<sup>t</sup>Bu) group (Fig. 2.5).



**Fig. 2.5. Standard cysteine protecting groups used in Fmoc methodology**



The Acn group can be removed by reagents such as metal ions,  $\text{Hg}^{\text{II}}$  and  $\text{Ag}^{\text{I}}$ , which give the reduced peptide, and iodine, which removes the protecting group with concomitant oxidation to the disulphide bridged peptide.

The pure cysteine-protected peptide was obtained by preparative HPLC followed by lyophilisation. Acn removal was initially attempted using  $\text{Hg}^{\text{II}}$  ions. The peptide was dissolved in 30% acetic acid and stirred overnight with 40 equivalents of mercuric acetate.  $\beta$ -mercaptoethanol was added to remove the mercury ions and the mixture filtered and chromatographed. However, the recovered material was exclusively the *bis*-Acn protected peptide.

The second reagent tried was cyanogen iodide, which has been found both to remove Acn protecting groups and to oxidise the peptide in a single step. The peptide was stirred with 50 equivalents of ICN in 50% aqueous methanol, in the dark, overnight, the reaction being quenched by the addition of sodium thiosulphate. Analysis of the recovered material revealed that the major portion was *bis*-Acn-protected peptide, with a very small amount of *mono*-Acn and several unidentified products.

Treatment with silver trifluoromethanesulphonate (silver triflate) resulted in a product which was exclusively the fully deprotected peptide; however, the recovery was initially very poor and required considerable optimisation. The reaction was carried out in the dark. The peptide and 20 equivalents of silver triflate were cooled on ice before the addition of TFA. The reaction was followed by HPLC and the optimum reaction time found to be 2.5 hours. Most of the TFA was then removed *in*



*vacuo* and a solution of dithiothreitol (DTT) (100 equivalents) added to liberate the peptide from its silver salt. In standard methodology the peptide silver salt is precipitated by the addition of ether before being redissolved in DTT in 50% aqueous acetic acid. However, due to the low solubility of the peptide, it was found that the yield was improved if the peptide silver salt was not precipitated. As has been mentioned previously, the solubility of the peptide is greater in basic, rather than acidic, solvents. Therefore the DTT was dissolved in tris buffer at pH8 and added to the slurry produced by removal of most of the TFA. It was thought that the peptide might be adhering to the glassware; therefore the flask was silylated prior to the start of the reaction, and, on removal of the TFA *in vacuo*, care was taken to avoid evaporating the flask to dryness. The peptide was stirred in the DTT solution overnight, which resulted in the formation of a yellow precipitate, the silver-DTT complex. This was removed by centrifugation and the peptide in the supernatant was purified directly by preparative HPLC. To improve recovery the pellet was resuspended in 6M guanidine HCl, brought to pH8 by the addition of tris base, and recentrifuged, the supernatant again being subjected to preparative HPLC. This procedure was repeated, but analytical HPLC of the supernatant showed that no further peptide had been liberated from the pellet. By using this optimised procedure the yield of fully deprotected peptide was approximately 50%.

Disulphide formation was carried out by a simple air oxidation in ammonium acetate buffer.

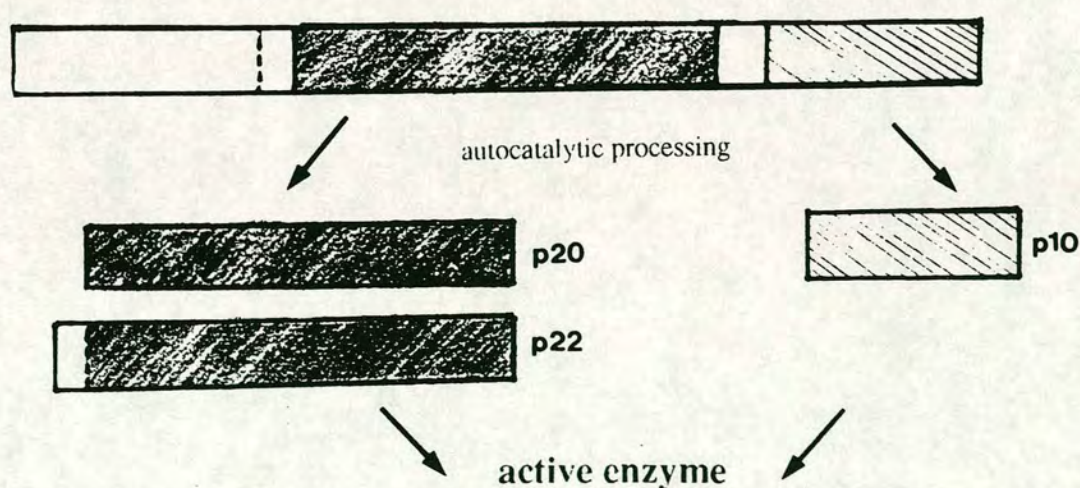




## 2.4. The Pro-region of ICE

Many enzymes, like ICE, are expressed as proenzymes, which undergo proteolytic processing to give the active species. For such enzymes the pro-region can be important for direction of the correct folding of the molecule. Additionally it may be that the cell stores certain enzymes in their precursor state, processing them only as required, thus allowing a rapid response to external influences. For some enzymes, for example subtilisin, it has been shown that the pro-region, once it has been cleaved from the mature enzyme, acts as an inhibitor of the enzyme itself<sup>98</sup>. Such a mechanism might be important in the feedback control of enzyme activity.

The pro-region of ICE is 119 amino acids long, and itself contains a secondary cleavage site, between Asp103 and Ser104, which gives rise to the alternatively processed fragment, p22, which, like p20, can combine with p10 to give an active enzyme (Fig. 2.6).



**Fig. 2.6. Alternative processing of proICE**



To try to test the theory that the pro region might have inhibitory activity against ICE, it was decided to synthesise the entire 119 amino acid sequence. This would also give an opportunity to try out methodology for the synthesis of large peptides being developed in this laboratory.

The initial plan was to synthesise the peptide by the fragment condensation of four smaller peptides, ranging in size from 22 to 45 residues (Fig. 2.7). This would have the added advantage that the shorter peptides could be tested individually for activity against the enzyme, thus showing whether any inhibitory activity resided in one particular linear segment of the pro-region, or within the peptide as a whole.

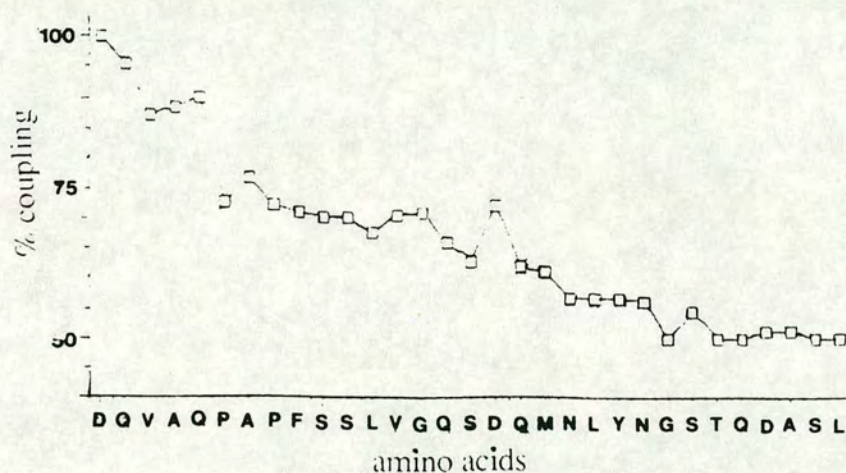
- a) 1-20  
**MADKVLKEKRKLFIRSMGEG**
- b) 21-65  
**TINGLLDELLQTRVLNKEEMEKVKRENATVM**  
**DKTRALIDSVIPKG**
- c) 66-88  
**AQACQICITYICEEDSYLAGTLG**
- d) 89-119  
**LSADQTSGNYLNMQDSQGVLSFPAPQAVQD**

**Fig. 2.7. Proposed fragments for the synthesis of the ICE pro-region**

The fourth fragment, 89-119, was synthesised by standard methodology on Wang resin (KU11). The synthesis was somewhat disappointing, with an overall coupling efficiency of just 50%. However, the cleavage and purification were straightforward, so that a reasonable yield was obtained. Examination of the deprotection profile for the synthesis showed that the poor couplings occurred at <sup>8</sup>Gly, <sup>26</sup>Pro and <sup>29</sup>Val (Fig. 2.8), residues immediately following Asn and Gln residues. In this synthesis, Asn and Gln were coupled as their side-chain-unprotected species. As it is known that the

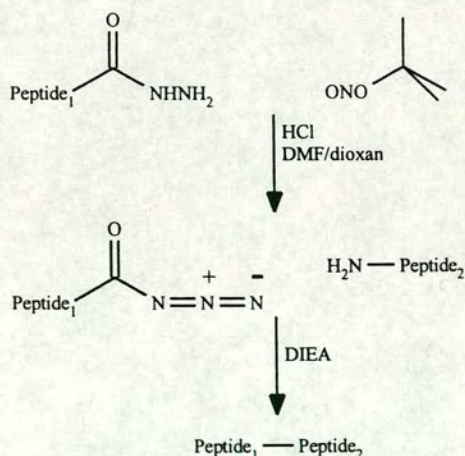


use of side-chain-unprotected Asn and Gln can sometimes result in poor coupling of subsequent residues, it was decided that, in future ICE pro-region syntheses, side-chain protection would be used.



**Fig. 2.8. Deprotection profile for KU61 - fourth fragment of the ICE pro-region**

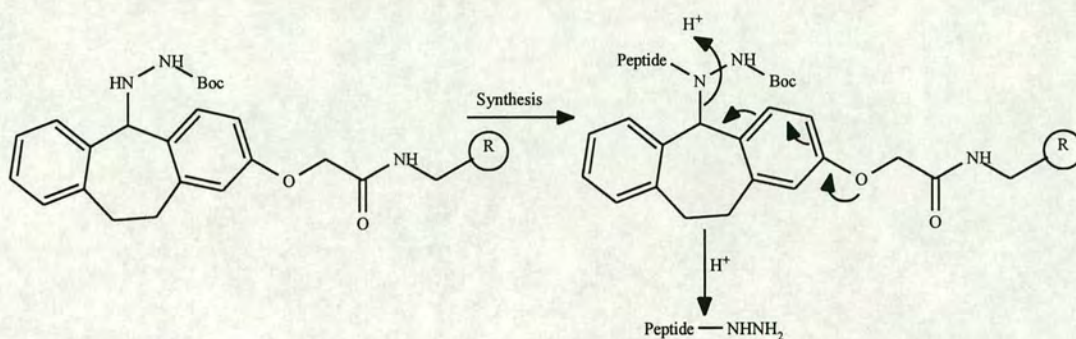
In this laboratory, chemical fragment condensations are carried out through the coupling of a peptide azide to the free N-terminal amine of a second peptide chain. The peptide azide is generated from a peptide-hydrazide by treatment with *t*-butyl nitrite in dry DMF with anhydrous HCl/1,4-dioxan (the Honzl-Rudinger method) (Fig. 2.9).



**Fig. 2.9. Chemical fragment condensation**

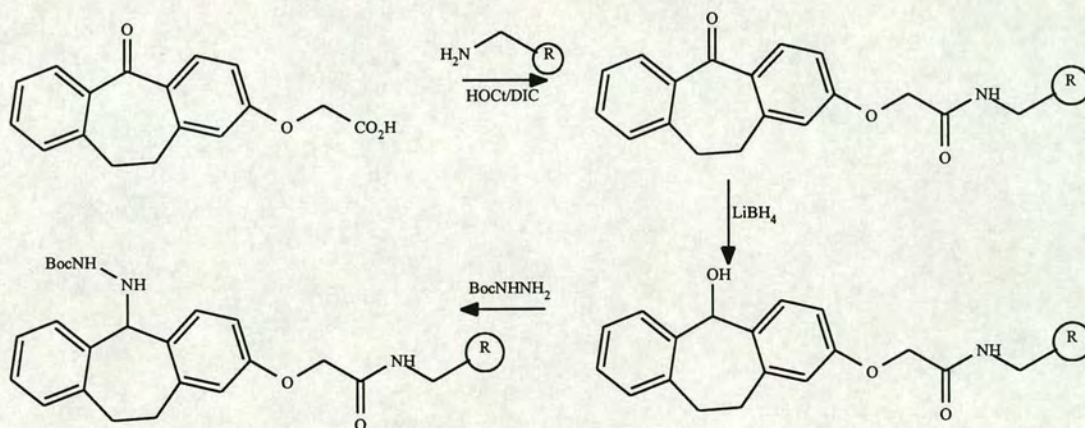


The required peptide-hydrazides can be generated directly on cleavage from the resin provided that the amino acid chain is built onto an appropriate linker. Such a linker has been developed in this laboratory<sup>99</sup> (Fig. 2.10).



**Fig. 2.10. Synthesis of a peptide-hydrazide using a dibenzosuberane linker**

The linker is coupled to aminomethyl resin in the form of a ketone. It is then reduced to the alcohol and treated with *t*-butylcarbazate to give the required product (Fig. 2.11).

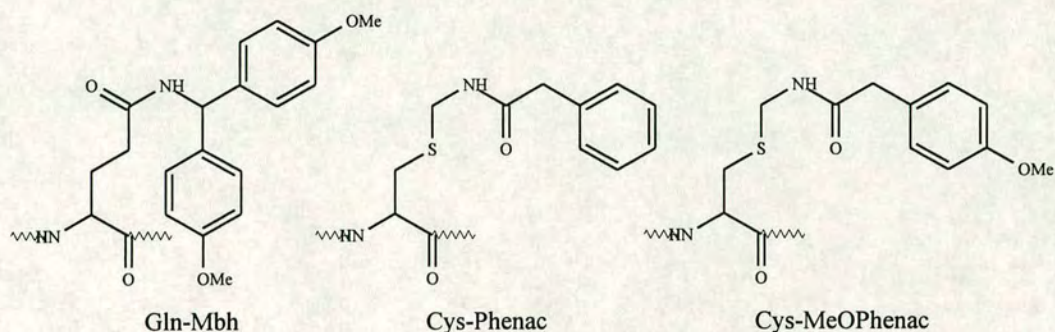


**Fig. 2.11. Preparation of hydrazide resin**

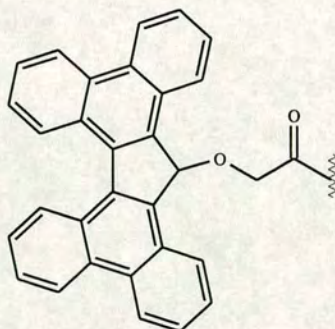
The third fragment, 66-88NHNH<sub>2</sub> (KU12), was synthesised using Fmoc-Gly-hydrazide resin prepared and loaded by David Pallin. The Gln residue was protected using the 4,4'-dimethoxybenzhydryl (Mbh) group, whilst the Cys residues were



protected using a recently developed group, phenylacetamidomethyl (Phenac)<sup>100,101</sup>, which can be removed by the enzyme penicillin acylase.



The deprotection profile showed a large drop in coupling efficiency for the coupling of <sup>11</sup>Ile, the residue following the first Cys-Phenac moiety. Therefore <sup>3</sup>Ala and <sup>6</sup>Ile, which follow the remaining cysteines, were given extended coupling times.



(18)

An initial attempt was made to purify KU12 using the TbFmoc (17-tetrabenzo[*a,c,g,i*]fluorenylmethoxycarbonyl) group<sup>102,103</sup> (18). This affinity-based N- $\alpha$  protecting group is coupled to the fully synthesised peptide before cleavage from the resin. The group has a high affinity for porous graphitised carbon (PGC). Therefore the required peptide, labelled at the N-terminus with TbFmoc, can be



separated from the deletion peptides by passing a solution of the crude peptide down a PGC column. The deletions are washed straight through whilst the full-length peptide adheres to the column. Because of its structural similarity to Fmoc, TbFmoc can be cleaved from the peptide by a base such as piperidine. Therefore the purified peptide is recovered from the PGC column by washing with 10% piperidine in, say, 50% iso-propanol/6M aqueous guanidine HCl.

Although a TbFmoc loading test showed that the group had been coupled satisfactorily to the resin-bound peptide, analytical HPLC, with monitoring at 364nm, showed little TbFmoc-peptide. (TbFmoc has a characteristic strong absorbance at this wavelength.) It appeared that the peptide itself was quite insoluble, and that the addition of the very hydrophobic TbFmoc group exacerbated this. Thus very little of the TbFmoc-peptide was getting into solution and it therefore could not be detected by HPLC. Clearly the proposed affinity chromatography will not work unless the crude peptide can be fully solubilised. Straightforward preparative HPLC of the non-TbFmoc crude peptide resulted in very poor recovery, suggesting that insolubility was a problem even for the free amine.

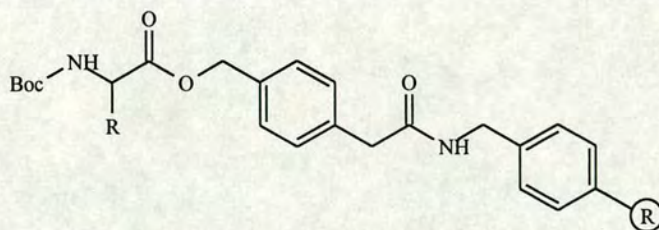
It was decided to resynthesise the peptide, but to change the cysteine-protecting group to 4-methoxyphenylacetamidomethyl (MeOPhenac). Ordinary TFA cleavage leaves the entire protecting group intact, but subsequent treatment with trifluoromethanesulphonic acid (TFMSA) should remove the methyl group, giving



improved solubility characteristics to the peptide and making the protecting group more susceptible to enzyme cleavage compared with Phenac<sup>101,104</sup>.

However, before this repeat synthesis could be carried out, a new batch of hydrazide resin had to be prepared. The preparation was carried out as previously, using aminomethyl resin obtained from another source. However, on attempting to carry out an automated synthesis it was found that the resin contained a large quantity of “fines”, which blocked the filters, making the synthesis impossible. A similar problem was encountered with subsequent batches of resin from different commercial sources. Clearly a consistent supply of resin was hard to obtain and therefore we decided to try a different resin system.

A quantity of PAM resin, preloaded with various N- $\alpha$  Boc-protected amino acids, was available in the laboratory (Fig. 2.12).



**Fig. 2.12. Boc-amino acid-PAM Resin**

Therefore, after removal of the Boc group by treatment with 90% TFA/H<sub>2</sub>O, the linker was loaded onto this resin, by overnight treatment with the HOCT activated ester. Quantitative Kaiser tests showed that this coupling proceeded in >99.5% yield. Under the procedure for aminomethyl resin, the ketone is reduced to a hydroxyl function using LiBH<sub>4</sub> (6 equivalents) in refluxing THF. However, these conditions



were found to be too harsh for use with the PAM resin since the reducing agent had a tendency to attack the ester function also, resulting in loss of the linker. Therefore the conditions were modified to use a single equivalent of  $\text{LiBH}_4$  at room temperature. In order to optimise the coupling it was found that reaction with *t*-butylcarbazate should be carried out as soon after the reduction as possible. The coupling of the Boc-hydrazine proceeded smoothly.

In order to test the resin, a 5-mer, LIFAG-NHNH<sub>2</sub> (KU13), was synthesised manually using bubbler apparatus. The synthesis went well, and, as expected, analytical HPLC of the crude cleaved peptide showed a single product which gave the correct molecular weight by FAB mass spectrometry.

At this stage, however, time was running short. Therefore it was decided to attempt the synthesis of the entire ICE pro region, linearly.

A trial synthesis (KU14a) was carried out on a 0.1mmole scale, ie. the peptide was synthesised rapidly using single coupling (symmetrical anhydride) throughout. This enables any problem areas of the sequence to be identified so that appropriate action can be taken during the repeat synthesis. Encouragingly, the trial synthesis finished with an overall coupling efficiency of 25% (see Fig. 2.13). Four areas were identified where coupling appeared to be problematic; therefore, during the second synthesis (KU14b), extended coupling times were used for these residues.



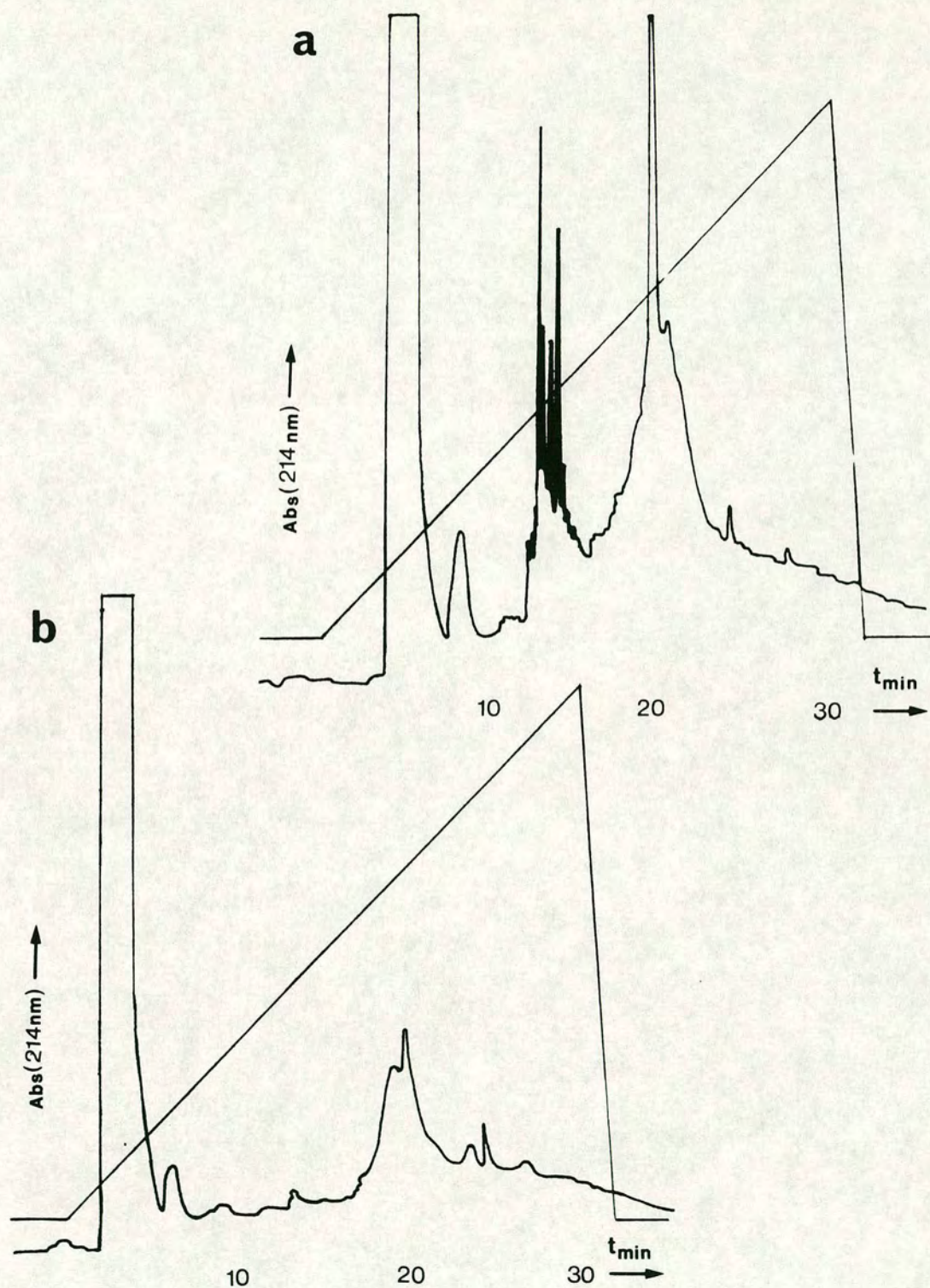




The largest drop in coupling efficiency was seen between  $^{63}\text{P}$  and  $^{62}\text{I}$ . It was decided that some of the resin (approximately one third) should be removed after the coupling of  $^{63}\text{P}$  (marked by an arrow in Fig. 2.13). This would allow the isolation of the C-terminal half of the pro region so that it could be tested alone for inhibitory activity against ICE. Moreover, by reducing the quantity of resin in the reaction vessel, the remaining peptide chain would be subjected to a greater number of equivalents of each activated amino acid, thus increasing the likelihood of a good coupling. In addition to the actual deprotection profiles as recorded during the syntheses, the lower part of Fig. 2.13 also shows an estimate of the actual coupling percentages, i.e. the values which would have been recorded had a third of the resin not been removed.

Inspection of the deprotection profiles shows an erratic rise and drop in the coupling percentages between  $^{104}\text{S}$  and  $^{99}\text{L}$  which is reflected in the two sets of data. Such abnormal changes in the apparent coupling can be caused by variations in the swelling of the resin which depend on the length and sequence of the growing peptide chain. The extended series of coupling percentages of  $>100\%$  seen for KU14a is probably due to a solvent delivery fault: if a smaller volume of 20% piperidine/DMF than normal is delivered, the resulting concentration of the Fmoc-piperidine adduct in the solution sent to the UV detector will be higher than it should be, resulting in an abnormally large peak area, and hence an overestimate of the coupling efficiency.





**Fig. 2.14. Purification of KU151 using TbFmoc/PGC methodology**

**a) before and b) after PGC treatment**

Column C; FR 1ml/min; Loop 2ml



TbFmoc was coupled to the completed peptide as normal. After cleavage the full length peptide was separated from the deletion products by treatment with porous graphitised carbon (PGC). After washing the PGC column several times to ensure that all the deletions were removed, the required peptide was washed from the column with 10% piperidine in *iso*-propanol/6M guanidine HCl (1:1). The solution was then desalted by gel filtration to give the cysteine-protected peptide.

Initially the resin cleavage was carried out using aqueous TFA conditions for 6 hours. Lyophilisation gave a jelly-like peptide which was extremely insoluble, suggesting that the peptide had not been fully deprotected. The cleavage protocol was changed to a two-step procedure in which the resin was first cleaved for 4 hours using the anhydrous trimethylsilyl bromide (TMSBr) cleavage mixture; then, following work-up, the resin and peptide were returned to the flask and treated with aqueous TFA for a further 2 hours. Amino acid analysis of the peptide obtained by this method suggested that it was the required product. However, a TOF MS gave a value of 15507.8, considerably higher than expected (13661.7 for the Cys-protected 119-mer). This suggested that the Asn and Gln groups had not been fully deprotected, with an average of 8 Mbh groups remaining on each molecule. Since TMSBr conditions are known to be the best for the removal of Mbh groups, the cleavage protocol was changed once again, to a single-step, 6-hour, TMSBr reaction. The resultant product was more soluble than the products of the other two cleavage methods, suggesting that the peptide might well be fully deprotected. Unfortunately it has proved impossible to confirm this by TOF MS, because the peptide is difficult



to handle and does not give good spectra, despite numerous attempts using different matrices, etc.

The peptide obtained from the PGC column gave rather a broad peak by analytical HPLC. It was thought that this might be due to partial removal of the cysteine protecting groups by the rather harsh cleavage conditions. It was decided to attempt to remove the cysteine protection to see whether any sharpening of the HPLC profile occurred, and to try to improve the solubility characteristics of a still somewhat insoluble peptide. The deprotection was carried out by treatment of the peptide with  $\text{Hg}^{\text{II}}$  ions in 50% aqueous acetic acid. The HPLC profile following gel filtration and lyophilisation was still rather broad, but this was not surprising since such a large peptide will adopt multiple conformations. A quantitative Ellman's test showed that there were an average of 1.8 free thiols per molecule, suggesting either that the deprotection was not entirely successful, or that some disulphide bonds had been formed. The peptide was subjected to a glutathione shuffle, stirring in 1M tris buffer (pH8) containing 1M guanidine HCl and oxidised (0.3mM) and reduced (3mM) glutathione, but no change in the HPLC profile was seen over several days.

## **2.5. The Enzyme Assay**

IL-1 $\beta$  converting enzyme (ICE) was over-expressed in *E. coli* by Dr David Giegel (Parke-Davis Pharmaceuticals, Ann Arbor, MI) and was sent to Edinburgh on dry ice in the form of crude cell lysates.



Enzyme activity was measured with a fluorescence assay, employing the substrate AcYVAD-7-amino-4-methylcoumarin (AMC) (1). ICE cleaves specifically between the Asp residue and AMC moiety, resulting in an increase in fluorescence which can be measured against time. Such an increase could be clearly seen in control experiments involving only enzyme and substrate; however, the addition of test peptides, including those known to be substrates of ICE (e.g. KU84 and proIL-1 $\beta$  111-122 (DML124) synthesised by D Maclean<sup>45</sup>), resulted in no change in the recorded time course. This suggested that for some reason the peptides were not acting even as competitive substrates of the enzyme.

It was thought that other proteases present in the crude lysate might be destroying the test peptide. Therefore the assay was carried out using some affinity-column-purified enzyme (also provided by D Giegel). The peptides once again failed to show any substrate activity. Another possible reason for the failure of the peptides was that they are produced as their TFA salts. Since the TFA can coat the peptide backbone in an unpredictable manner, it was thought possible that it might be interfering with the interaction of the peptide with the enzyme. An attempt was made to desalt some of the peptides by gel filtration (G15 Sephadex) followed by dialysis. Again this had no effect on the assay results.

In order to try to ascertain whether any of the peptides were indeed acting as substrates, an HPLC assay was carried out. The peptide DML124 was incubated at



30°C for various times, then injected onto an HPLC column. Little change could be seen in the HPLC profile, even when the assay mixture was incubated overnight. In order to check that the products of enzyme cleavage would be clearly seen with the assay gradient used, the expected fragments, proIL-1 $\beta$  111-116 (KU15) and proIL-1 $\beta$  117-122 (KU16), were synthesised, and injected onto the assay column. This showed that they would be clearly visible, eluting earlier than the unproteolysed peptide.

These results were rather puzzling. Therefore it was decided to send the test peptides to our collaborators in the USA, to see whether more favourable results could be obtained. The enzyme inhibition assay used at Parke-Davis was essentially the same as that used here, except that the substrate used was AcYVAD-*p*-nitroaniline (2) with UV/vis detection. The results obtained showed that little or no inhibition could be observed with any of the test peptides (Table 2.3).

The assay is run in the presence of a dithiothreitol (DTT) concentration of 10mM, which is sufficient to disrupt most disulphide bonds; thus both KU9-oxidised and KU2-oxidised are almost certainly reduced by the assay buffer.

The crmA peptides, KU7 and KU9, do seem to show a slight inhibitory effect. However, as ICE is a cysteine protease, and both the test peptides contain free cysteine side-chains, the inhibition seen could be a nonspecific thiol effect.



<u>Peptides</u>	<u>% Enzyme Activity at 125µM Peptide Conc.</u>
KU2 - oxidised	
proIL-1β 113-122-CysProD.ValCys-160-163	100.05
KU3	
proIL-1β 113-122-ACPC-160-163	97.13
KU4	
proIL-1β 113-120	103.07
KU5	
proIL-1β 111-122 <sup>116</sup> Glu	107.23
KU6	
proIL-1β 111-122 <sup>119</sup> D.Val	105.61
KU7	
crmA 300-307	95.04
KU8	
crmA 300-307 <sup>304</sup> Ala <sup>305</sup> Cys	101.40
KU9 - reduced	
crmA 298-309	89.27
KU9 - oxidised	93.53
KU11	
ICE pro-region 89-119	106.09
KU14	
ICE pro-region 1-119	100.00

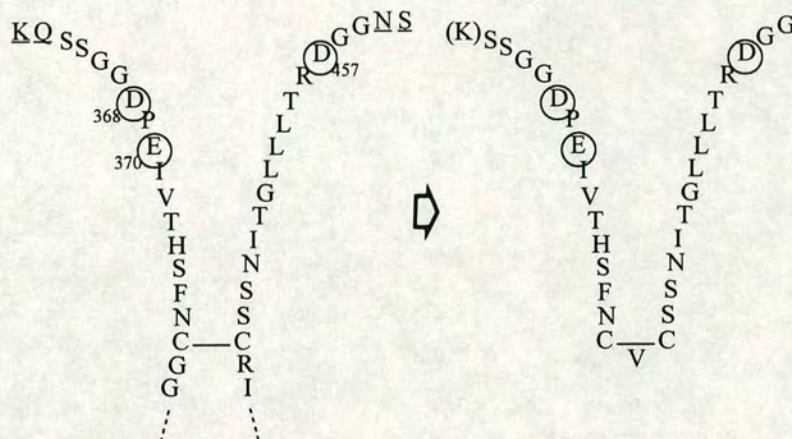
**Table 2.3. Enzyme assay results**

## **2.6. gp120 Peptides**

In order for the human immunodeficiency virus (HIV)-1 to infect CD4-positive lymphocytes, the virus envelope glycoprotein, gp120, must interact with CD4. Five gp120 residues important for CD4 binding have been identified by site-directed mutagenesis: these are Thr257, Asp368, Gly370, Trp427 and Asp457<sup>105</sup>. A 32-residue peptide, GC1, containing three of these residues, Asp368, Glu370 and Asp457, has been synthesised in this laboratory, and has been shown to act as a discontinuous epitope of gp120, and to bind to CD4<sup>106</sup>.



The design of GC1 utilises the fact that two cysteine residues close to the important Asp and Glu residues form a disulphide bridge in native gp120. GC1 consists of the relevant sequences from the conserved C3 and C4 regions of gp120, continuing as far as the cystine group. To facilitate formation of the disulphide bond, the two cysteine residues are separated by a valine residue (see Fig. 2.15).



**Fig. 2.15. The design of GC1**

The CysValCys moiety is believed to adopt the structure of a  $\gamma$ -turn, thus causing the peptide chain to fold back on itself. However formation of the disulphide bond requires oxidation with DMSO, relatively harsh conditions which can result in poor peptide recovery.

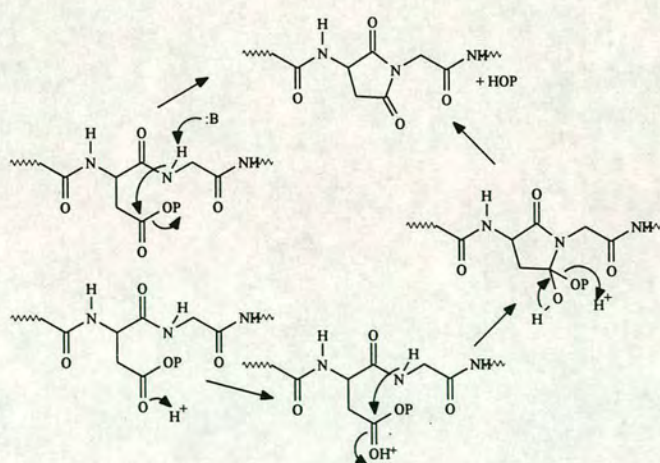
Following the successful use of the dipeptide ProD.Val to induce formation of a  $\beta$ -turn in KU2, it was decided to synthesise an analogue of GC1 in which the CysValCys grouping was replaced by CysProD.ValCys. As expected this modification allowed the disulphide to be formed by a simple, clean, air oxidation.



A second analogue of GC1 has been synthesised in which the entire  $\beta/\gamma$ -turn region has been replaced by the proposed  $\beta$ -turn mimic, ACPC. The use of this residue means that two steps in the synthesis of GC1 and the analogue KU17, namely deprotection of the cysteine residues and oxidation, can be eliminated. The result is that a higher yield of peptide can be obtained in a shorter time.

At the time of the synthesis of the ACPC analogue, KU18, a limited amount of FmocACPC was available. Therefore the synthesis was carried out on a slightly smaller scale than usual (0.2mmole) and, in order to ensure that coupling of the ACPC residue was as complete as possible, it was coupled manually, with sonication, overnight as the HOt activated ester.

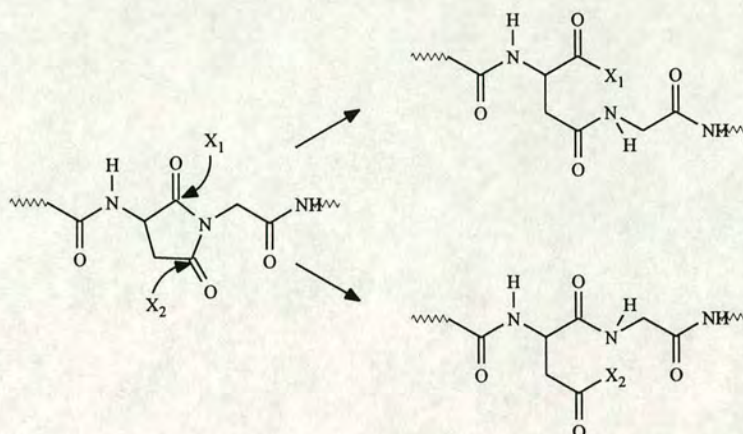
Optimisation of yield is extremely important for GC1 and its analogues due to the presence, near the N-terminal end of the peptide, of an Asp-Gly pair. Such pairs of residues are prone, under both acidic and basic conditions to undergo a cyclisation to form an aspartimide (Fig. 2.16).



**Fig. 2.16. Acid & base catalysed aspartimide formation**



The aspartimide can then be opened by nucleophilic attack to give either the required  $\alpha$ -peptide, or the unnatural  $\beta$ -peptide (Fig. 2.17).



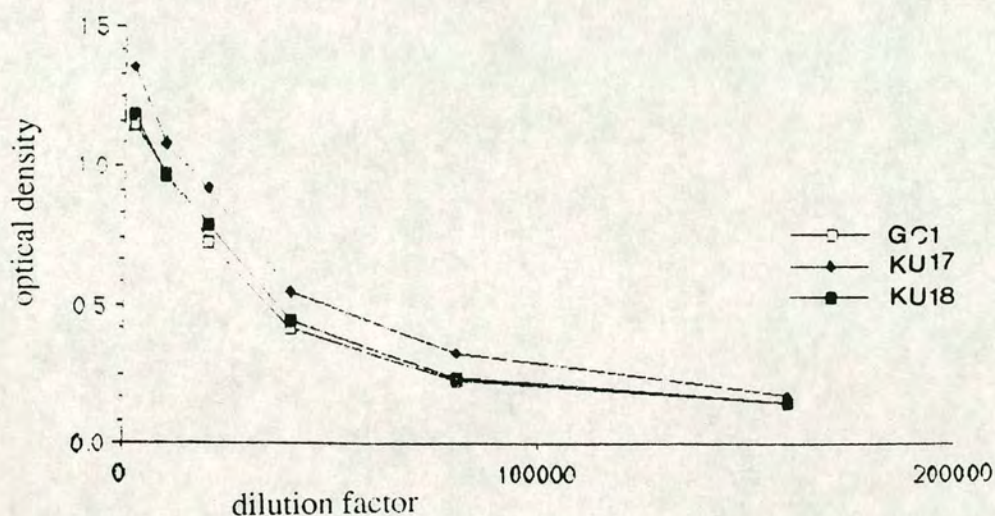
**Fig. 2.17. Nucleophilic attack on an aspartimide to give  $\alpha$ - and  $\beta$ -peptides**

Analytical HPLC of crude, cleaved GC1 and its analogues, KU17 and KU18, clearly shows that a significant proportion of the peptide has undergone aspartimide formation. The required peptide and the aspartimide-containing product elute very close together, thus making preparative HPLC very difficult and resulting in loss of yield. Clearly, then, if two chemical steps and concomitant losses in yield could be avoided, this would be advantageous.

The analogues, KU17 and KU18, have been tested for cross-reactivity with the polyclonal antibodies raised against GC1 by Graham Cotton. The peptides, GC1, KU17 and KU18, were bound to plates and treated with varying dilutions of anti-GC1 serum. After washing, this was treated with a goat anti-mouse Ig(G,A,M)-horseradish peroxidase complex. Finally, treatment with tetramethylbenzidine in the presence of hydrogen peroxide resulted in a coloured solution, the optical density of which indicates the extent of anti-GC1 binding to the peptide<sup>108</sup>.



Interestingly, as can be seen from figure 2.18, the analogues have been shown to cross-react to a similar extent to GC1 itself. This suggests that KU17 and KU18 are at least capable of adopting the same conformations as GC1, though whether their conformations are predominantly the same has yet to be confirmed.



**Fig. 2.18. Comparison of the cross-reactivities of GC1 and analogues with anti-GC1 antibodies**

## 2.7. NMR

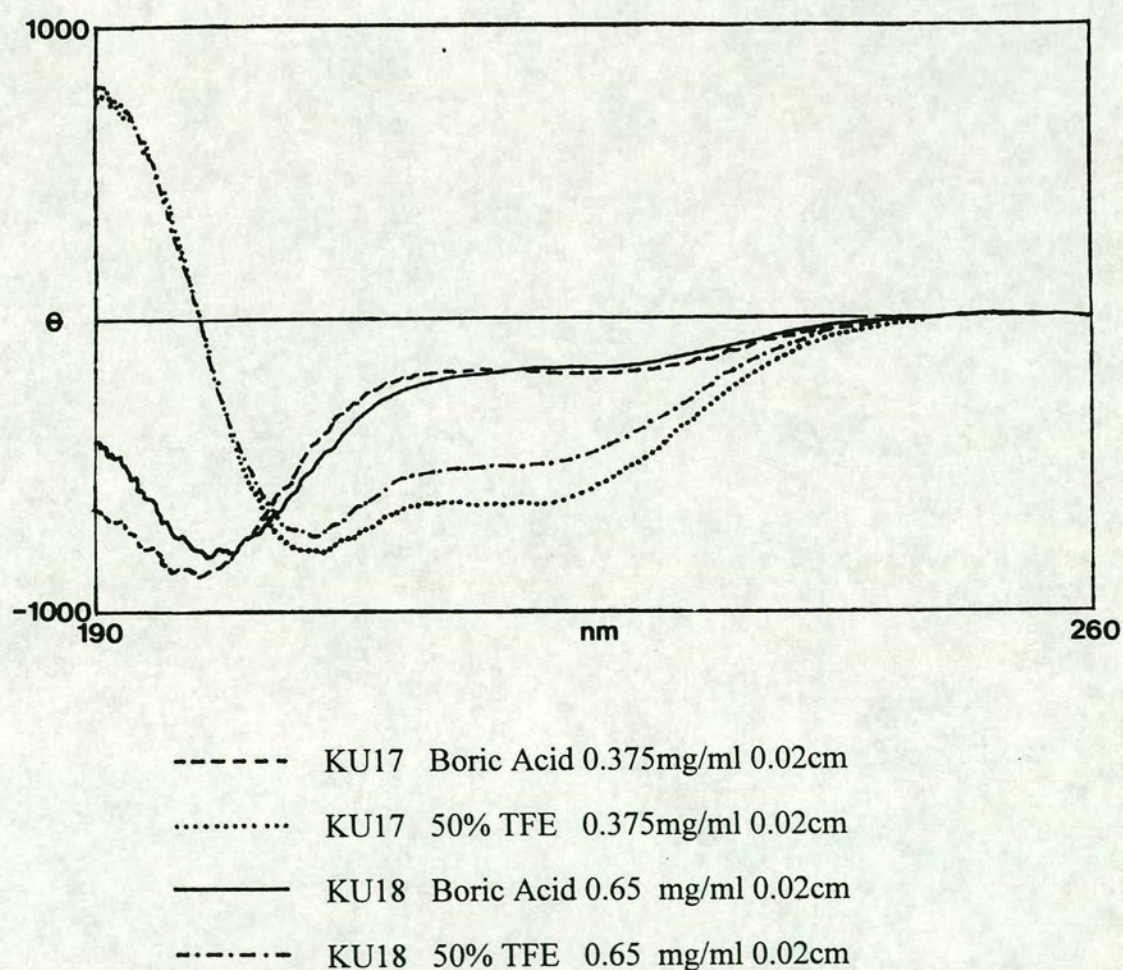
3D NMR studies were carried out on the 'β-turn'-containing peptides, those containing the CysProD.ValCys sequence, KU2 and KU17, and those containing the ACPC residue, KU3 and KU18. It was hoped that some β-sheet structure would be apparent. Whilst the DQ COSY and TOCSY spectra showed some dispersion, there was no evidence for NOESY cross peaks relating to residues other than those adjacent to one another, showing that these peptides do not adopt a well-defined structure in solution. Although this is somewhat disappointing, the peptides should nevertheless be capable of adopting the required β-sheet structure under the influence of an external agent, e.g., in the case of KU17 and KU18, binding to an antibody.



## 2.8. 3-Aminocyclopentane-1-carboxylic Acid as a $\beta$ -turn Mimic

The NMR data do not give any direct evidence of ACPC behaving as a  $\beta$ -turn mimic. However, peptides in which the  $\beta$ -turn formed by the dipeptide sequence ProD.Val is held in its conformation by a disulphide bond give similar NMR spectra to their ACPC analogues. Therefore the lack of NMR evidence for  $\beta$ -sheet structure cannot be taken as proof that ACPC is not, or is not capable of, acting as a  $\beta$ -turn mimic.

The circular dichroism (CD) spectra of the GC1 analogues, KU17 and KU18, are very similar (Fig. 2.19), indicating that they adopt similar solution-phase conformations, suggestive evidence for ACPC as a  $\beta$ -turn mimic.



**Fig. 2.19. CD spectra of KU17 and KU18**



It is to be expected, with a flexible  $\beta$ -turn mimic such as ACPC, that the degree to which it promotes turn formation is dependent upon the peptide sequence. This is clearly seen with a sequence from nerve growth factor (NGF) which was synthesised by Angela Kelly (AK20)<sup>97</sup>. The solution structure has been elucidated by NMR<sup>107</sup>. Thus it has been shown that the peptide does not adopt the predicted  $\beta$ -sheet structure, but rather the ACPC moiety appears to act as a hydrophobic core with the peptide backbone being wrapped around it.

In conclusion, it cannot be positively said that ACPC acts a  $\beta$ -turn mimic; however, it may do so where the surrounding peptide sequence has a propensity to form a  $\beta$ -sheet structure.



- KU1** proIL-1 $\beta$  113-122 CysGlyGlyCys 160-163  
YVHDAPVRSLCGGCMSEFV
- KU2** proIL-1 $\beta$  113-122 CysProDValCys 160-163  
YVHDAPVRSLCPdVCMSFV
- KU3** proIL-1 $\beta$  113-122 (ACPC) 160-163  
YVHDAPVRSL(ACPC)MSFV
- KU4** proIL-1 $\beta$  113-120  
YVHDAPVR
- KU5** proIL-1 $\beta$  111-122 <sup>116</sup>Glu  
EAYVHEAPVRSL
- KU6** proIL-1 $\beta$  111-122 <sup>119</sup>D.Val  
EAYVHDAPdVRSL
- KU7** CrmA 300-307  
Ac LVADCAST NH<sub>2</sub>
- KU8** CrmA 300-307 <sup>304</sup>Ala<sup>305</sup>Cys  
Ac LVADACST NH<sub>2</sub>
- KU9** CrmA 298-309  
Ac CALVADCASTVT NH<sub>2</sub>
- KU10** CrmA 297-309  
Ac TCALVADCASTVT NH<sub>2</sub>
- KU11** proICE 89-119  
LSADQTSIGNYLNMQDSQGVLSSEFPAPQAVQD
- KU12** proICE 66-88  
AQACQICITYICEEDSYLAGTLG NHNH<sub>2</sub>
- KU13** LIFAG NHNH<sub>2</sub>
- KU14** proICE 1-119
- KU15** proIL-1 $\beta$  111-116  
EAYVHD
- KU16** proIL-1 $\beta$  117-122  
APVRSL
- KU17** HIV<sub>III</sub>B gp120 Lys 364-378 ProD.Val 445-459  
KSSGGDPEIVTHSFNCPdVCSSNITGLLLTRDGG
- KU18** HIV<sub>III</sub>B gp120 Lys 364-377 (ACPC) 446-459  
KSSGGDPEIVTHSFN(ACPC)SSNITGLLLTRDGG



### 3. Experimental

#### 3.1 Notes

All Fmoc-amino acids were purchased from Novabiochem, Bachem or Raylo, with the exception of Fmoc-*cis*-3-aminocyclopentane-1-carboxylic acid (FmocACPC), FmocCys(Phenac) and FmocCys(MeOPhenac), which were prepared in this laboratory by the methods described in this text, or of A Brown<sup>101</sup>. Optically pure *cis*-3-aminocyclopent-4-ene-1-carboxylic acid was obtained from Chiroscience. All amino acids are of the L-configuration, unless otherwise stated. Peptide synthesis grade trifluoroacetic acid (TFA) was obtained from Applied Biosystems. Peptide synthesis grade dimethylformamide (DMF) and 1,4-dioxan, and HPLC grade acetonitrile, were obtained from Rathburn Chemicals. Automated peptide syntheses were carried out on an ABI 430A peptide synthesiser. UV spectra were recorded on a Varian Cary 210 double beam spectrophotometer. Sonication was carried out in a Decon FS300b sonic bath. NMR spectra were recorded on Bruker WP200 (200MHz), Bruker WH-360 (360MHz) and Varian VXR5000 (600MHz) instruments; chemical shifts were measured relative to TMS assigned to zero. Fast atom bombardment mass spectra (FAB MS) were measured on a Kratos MS50TC machine, electron ionisation mass spectra (EI MS) on a Kratos 902MS, and time of flight (TOF MS) on a Perspective Biosystems LaserTec Benchtop II laser desorption system from Vestec mass spectrometry products. Circular dichroism spectra were recorded in Stirling on a JASCO J600 spectropolarimeter. Fluorescence spectra were



recorded on a Perkin-Elmer LS50 luminescence spectrometer fitted with a Grant LTD-6 thermostatic control. Infrared spectra were recorded on a BIO-RAD SPC3200 instrument. Optical rotations were measured using an AA1000 polarimeter (Optical Activity Ltd). Elemental analyses were performed on a Perkin-Elmer 2400 CHN elemental analyzer. Melting points were recorded in open capillaries on a Buchi 510 oil immersion melting point apparatus, and are uncorrected. Throughout the text, ether refers to diethyl ether and dioxan to 1,4-dioxan.

## **3.2. Solid Phase Peptide Synthesis**

### **3.2.1. Side-chain Protecting Groups in Fmoc SPPS**

The following amino acids are coupled without side-chain protection:

Ala, Gly, Ile, Leu, Met, Phe, Pro, Trp, Val.

The following are coupled with side-chains protected as <sup>t</sup>Bu esters:

Asp, Glu.

The following are coupled with side-chains protected as <sup>t</sup>Bu ethers:

Ser, Thr, Tyr.

Arg is protected by the pentamethylchromansulphonyl (Pmc) group, His by the trityl (Trt) group and Lys as a urethane by the *t*-butyloxycarbonyl (Boc) group.



The remaining residues, Cys, Asn and Gln, may be coupled in a variety of forms, the choice of protecting group being dependent on the sequence and/or synthetic strategy. Cys is commercially available as Cys(Trt), Cys(S<sup>t</sup>Bu) and Cys-acetamidomethyl (Acm) derivatives. The trityl group is removed under standard TFA cleavage conditions, whilst the other two groups are stable to TFA and are removed subsequently under specialized conditions. Two other TFA stable Cys protecting groups have been used in this work, phenylacetamidomethyl (Phenac) and 4-methoxyphenylacetamidomethyl (MeOPhenac), the Cys derivatives being prepared in this laboratory. Asn and Gln may be coupled with no side-chain protection, or may be protected by Trt or 4,4'-dimethoxybenzhydryl (Mbh) groups. In the case of these variable amino acids, the protecting group used in each synthesis is specified in the text.

### **3.2.2. Loading Wang Resin for Peptide-acid Synthesis**

The first amino acid residue is coupled to the resin via its symmetrical anhydride.

Fmoc-amino acid (6 equivalents) and DIC (3 equivalents) are stirred in DMF (5ml) for 15 minutes. Wang resin and a catalytic amount of DMAP (approximately 8mg) are added. Extra DMF may be added if required, to fully swell the resin. The mixture is sonicated for two hours at room temperature. The resin is separated by filtration and washed with copious DMF, dioxan and DMF. Finally the resin is dried by washing with ether.



If a low resin loading is required the procedure may be modified as follows; 1.5 equivalents of the symmetrical anhydride may be used (hence 3 equivalents of Fmoc-amino acid and 1.5 equivalents of DIC), and the mixture may be sonicated for just one hour.

When synthesising peptide-amides, manual loading of the first amino acid is unnecessary; the whole chain assembly may be carried out automatically.

### **3.2.3. The Fmoc Loading Test**

Dry Fmoc-amino acid resin (2-3mg), accurately weighed, is sonicated in 20% piperidine/DMF (10ml) for 10 minutes. The resin is removed by filtration and the UV spectrum of the filtrate recorded between 340 and 280nm. The percentage loading is calculated from the absorbance of the piperidine-Fmoc adduct at 302nm, by a computer program using the Beer-Lambert law, calibrated against known quantities of Fmoc-Gly-OH. The average of two such tests determines the resin loading.

### **3.2.4. Automated Peptide Synthesis**

A single cycle of an automated synthesis, resulting in the coupling of a single residue, comprises the following steps: **1. capping**, to block any unreacted free amines, which would result in the synthesis of a peptide missing one of its internal



residues; **2. deprotection**, to remove the N- $\alpha$  protecting group (Fmoc) in preparation for the coupling of the next amino acid; **3. coupling**, either once, twice or, exceptionally, three times (Fig 3.1).

### 1. Capping

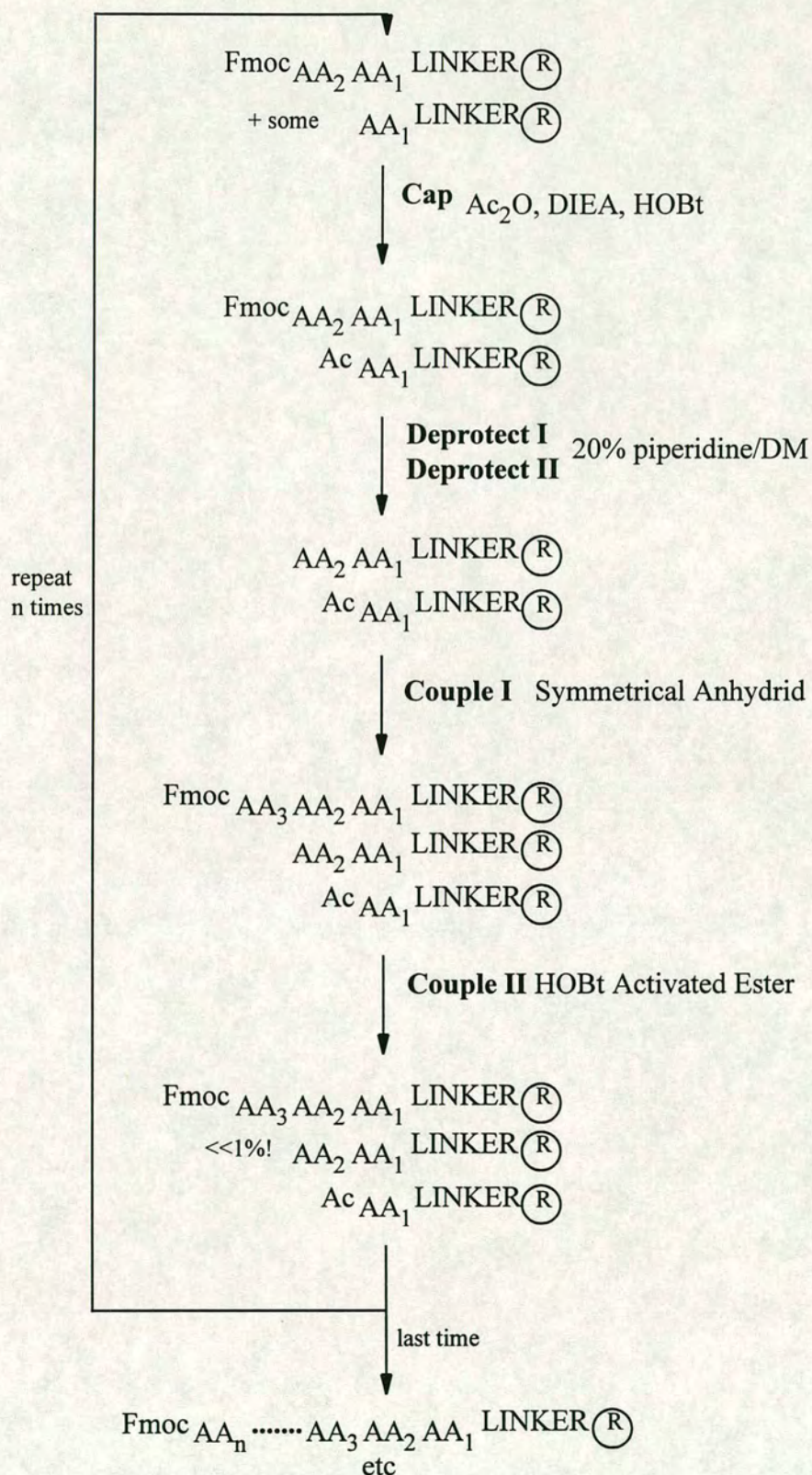
The resin is shaken with 0.5M acetic anhydride, 0.125M DIEA and 0.2% HOBt in DMF (10ml) for 10 minutes. The capping solution is then drained from the reaction vessel and the resin washed with 6 portions of DMF.

### 2. Deprotection

The resin is shaken with 20% piperidine/DMF (10ml) for 3 minutes. It is drained, washed four times with DMF, and then shaken with further 20% piperidine/DMF (10ml) for 1 minute. Finally the resin is washed with 6 portions of DMF/dioxan (1:1).

The percentage of amino acid successfully coupled in the preceding cycle is determined by analysis of an aliquot of the deprotection filtrate. The UV absorbance of the sample at 302nm is measured by an ABI 758A detector, with on-line integration. Comparison of the area of each successive deprotection peak with that of the first amino acid allows an estimate of the percentage coupling of each residue to be made.





**Fig. 3.1.** Schematic showing the key stages in an automated double couple cycle.



### 3. Coupling

The standard double couple cycle used for most syntheses consists of one coupling using 2 equivalents of symmetrical anhydride, followed by a second with 2 equivalents of HOBt activated ester. The exceptions to this are asparagine, glutamine and histidine, which are coupled twice as the HOBt activated ester (never the symmetrical anhydride), and glycine, which is single coupled using 4 equivalents of the symmetrical anhydride. In the synthesis of large peptides (>50 residues) this procedure is modified, the second coupling being carried out with 4 equivalents of activated ester. When, exceptionally, a residue is coupled three times, a symmetrical anhydride coupling is followed by two HOBt activated ester couplings (for the standard residues).

Both the symmetrical anhydride and the activated ester are preformed in an 'activation vessel' before being transferred to the reaction vessel. For symmetrical anhydride formation, 1mmol of Fmoc-amino acid is treated with 0.5mmol of DIC in DMF/dioxan (1:1, 8ml) for 10 minutes, whilst the activated ester is formed by the reaction of 0.5mmol Fmoc-amino acid, 0.5mmol HOBt and 0.5mmol DIC, also in DMF/dioxan (1:1, 8ml) for 20 minutes. In both cases coupling is allowed to proceed for 30 minutes in a standard cycle. When extended coupling times are used the SA is coupled for 1 hour and the AE for 1.5 hours.

The resin is washed four times.



#### 4. The end cycle

Once chain assembly is complete another capping step is carried out. The final Fmoc group may be left on the peptide and removed manually later. Alternatively the Fmoc may be removed by a final deprotection step. The peptide may be left with a free N-terminal amine, or, if an acetylated peptide is required, a further capping step may be carried out.

#### **3.2.5. Manual Peptide Synthesis**

The procedure for manual peptide synthesis using bubbler apparatus (see Fig 3.2) is essentially the same as described above for automated synthesis. Activation of the amino acids is carried out in a separate round-bottomed flask. A single, 10 minute, deprotection step is used.

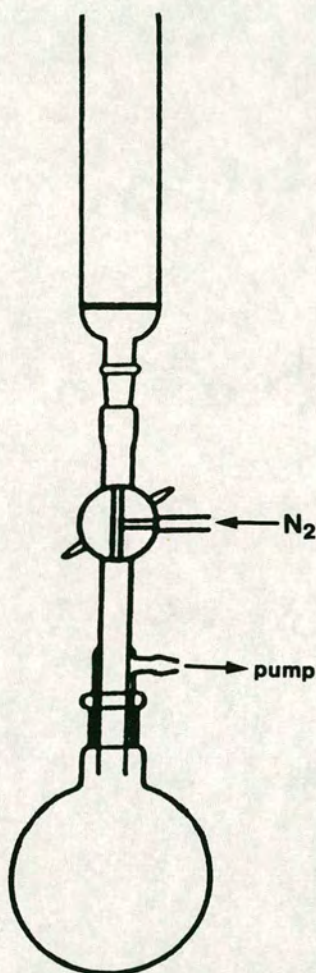
Monitoring of the coupling efficiency by UV is not carried out. Instead it is possible to ascertain whether complete coupling has taken place by carrying out a Kaiser test.

#### Kaiser Test

A few beads of damp resin are placed in a small vial. 76% w/w phenol/ethanol (75µl), 0.2mM potassium cyanide/pyridine (100µl) and 0.28M ninhydrin/ethanol (75µl) are added. The vial is placed in a heating block at >100°C for 7 minutes. If



free amine groups are present the solution and beads will show a blue tinge. However if the coupling is complete the solution will remain yellow and the synthesis may continue.



**Fig. 3.2. Bubbler Apparatus for the Manual Synthesis of Peptides.**

The peptide-resin is contained within the cylindrical reaction vessel, and is agitated with nitrogen. Reagents are added manually from the top and removed under suction through the glass sinter at the bottom of the reaction vessel. Waste solvents and excess reagents are collected in the lower reservoir.



### 3.2.6. Loading TbFmoc onto Peptide-resin

The loading of the last amino acid residue in the peptide chain is ascertained accurately by carrying out an Fmoc loading test (see section 3.2.2). Between 3 and 10 equivalents of TbFmoc chloroformate are dissolved in DCM (5ml). The resin is added, followed by an amount of DIEA equivalent to that of the TbFmocCl used. The mixture is sonicated in the dark for three hours. The resin is filtered and washed with DCM, then returned to the flask with fresh DCM and sonicated for a further 10 minutes. This procedure is repeated to ensure that all excess TbFmocCl is removed. The resin is dried by washing with ether; it is stored in dioxan at 4°C, in the dark.

All further manipulations of TbFmoc-peptides (cleavage, lyophilisation etc) must be performed in the dark.

### 3.2.7. The TbFmoc Loading Test

Dry TbFmoc-peptide-resin (1-3mg), accurately weighed, is sonicated in 20% piperidine/dioxan (2ml) for 10 minutes. The UV spectrum of the solution is recorded between 400 and 340nm. The percentage loading is calculated from the equation:

$$\% \text{ loading} = \frac{0.613 \times \text{Absorbance at } 364 \text{ nm}}{5 \times \text{weight of resin in mg}} \times 100$$

The average of two such tests determines the TbFmoc loading.



### 3.2.8. Acidolytic Cleavage from the Resin

Peptide-amides and peptide-acids are both cleaved and deprotected under the same conditions. Two methods are used, aqueous TFA cleavage and the anhydrous trimethylsilyl bromide (TMSBr) cleavage. In both cases it is necessary to include cation scavengers in the reaction mixture to prevent the attack of sensitive residues by the dislodged protecting groups.

#### Aqueous TFA Cleavage

The dry peptide-resin is placed in a small round-bottomed flask containing a magnetic stirrer bar and fitted with a nitrogen bubbler. The scavengers are added to the resin and the mixture is stirred for 10-15 minutes. (The choice of scavengers depends on the residues present; the scavengers which have been used during this work are specified in the text.) Water and TFA are added and the mixture is stirred under nitrogen at room temperature for a variable length of time, dependent upon the length of the peptide and the nature of the protecting groups.

#### Anhydrous TMSBr Cleavage

TFA (7.5ml), *m*-cresol (100μl), EDT (500μl) and thioanisole (1.17ml) are placed in a round-bottomed flask and cooled on ice for 30 minutes. TMSBr (1.3ml) is added and the mixture cooled for a further 5 minutes before the addition of the dry peptide-



resin. The mixture is stirred, with cooling on ice, under a slow stream of nitrogen, for a time dependent upon the nature of the peptide.

#### Work-up

The work-up is carried out in the same way for both cleavage methods. The resin is removed by filtration and washed with TFA. The volume of TFA is reduced *in vacuo* and the peptide triturated from the resultant dark oil by the addition of ether. The peptide is filtered on a glass sinter and washed with further ether. Finally it is dissolved from the sinter in aqueous acetic acid (usually 20% AcOH/H<sub>2</sub>O), and lyophilised to give the crude peptide.

#### **3.2.9. High Performance Liquid Chromatography**

Reverse phase HPLC for the analysis and purification of cleaved peptides is carried out using an ABI 1783A programmable detector, two ABI 140A solvent delivery pumps and an ABI 1408A injector. The columns referred to in the text are:

##### Analytical Columns

ABI Aquapore RP300 reverse phase silica (300Å pore size, 7µm spherical silica)

**A:** 220 x 4.6mm C<sub>18</sub>

**B:** 100 x 4.6mm C<sub>18</sub>

**C:** 220 x 4.6mm C<sub>8</sub>



Vydac reverse phase silica (300Å pore size, 5µm spherical silica)

**D:** 250 x 4.6mm C<sub>18</sub>

**E:** 250 x 4.6mm C<sub>8</sub>

#### Preparative Columns

ABI Aquapore RP300 (equivalent to analytical columns)

**F:** 110 x 9.2mm C<sub>18</sub>

**G:** 250 x 9.2mm C<sub>18</sub>

**H:** 110 x 9.2mm C<sub>8</sub>

**J:** 250 x 9.2mm C<sub>8</sub>

Vydac reverse phase silica (10µm particle size)

**K:** 250 x 22mm C<sub>18</sub>

The peptides are eluted by an increasing concentration of acetonitrile in water. The solvent system used is:

Solvent A: H<sub>2</sub>O/0.1% TFA

Solvent B: CH<sub>3</sub>CN/0.1% TFA

In this work, two gradients were used for analytical HPLC:

gradient I: (t,%B) (0,10)(30,90)(32,10)

gradient II: (t,%B) (0,10)(2,10)(32,90)(34,90)(36,10)

For preparative HPLC the gradient used was dependent on the nature and quantity of impurities present. The conditions used are specified in the relevant part of the text.

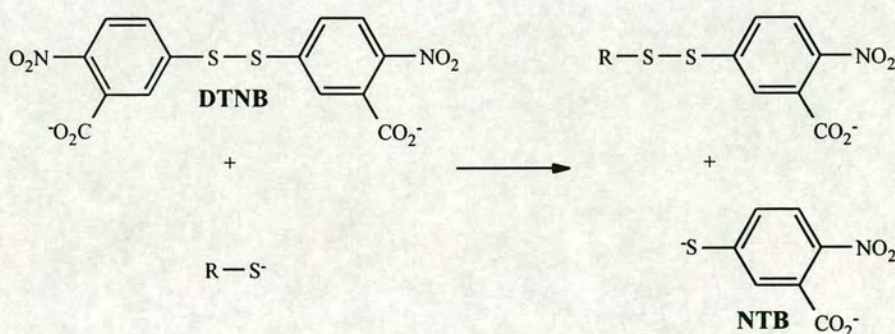


### 3.2.10. Amino Acid Analysis

Approximately 1mg of peptide is dissolved in approximately 2ml of 6M HCl in a Carius tube. The sample is degassed and the tube is sealed. Hydrolysis is carried out at 110°C for 24 hours. For large peptides (>50 residues) hydrolysis proceeds for 48 hours. The aqueous HCl is then removed *in vacuo*, and the hydrolysed material redissolved in sodium citrate buffer, pH2.2 (2ml) for analysis. This is carried out on an LKB 4151 amino acid analyser.

### 3.2.11. The Ellman's Test

The Ellman's test is used to reveal the presence or absence of free thiols. It may be performed either quantitatively, or qualitatively, when a bright yellow solution resulting from the production of the nitrothiobenzoate (NTB) chromophore indicates the presence of unprotected cysteines.



**Fig. 3.3 The Ellman's Test**

0.1-0.2mg of peptide are dissolved in 6M guanidine HCl with 0.1M phosphate buffer (pH7.3) and 1mM EDTA (2ml). 100µl of a 3mM solution of 5,5'-dithiobis(2-



nitrobenzoic acid) (DTNB) in 0.1M phosphate buffer (pH7.3) is added, and the solutions thoroughly mixed by shaking. Colour development is rapid.

For a quantitative test the absorbance of the protein solution is measured at 412nm. Knowing that the extinction coefficient of the NTB anion in 6M guanidine HCl is 13700/M cm, the molar concentration of free thiols can be calculated.

### **3.3. Experimental**

#### **KU2 proIL-1 $\beta$ 113-122-Cys-Pro-D.Val-Cys-160-163**

**Tyr Val His Asp Ala Pro Val Arg Ser Leu Cys Pro D.Val Cys Met Ser Phe Val**

The synthesis was carried out on a 0.25mmole scale, starting with FmocVal-resin (0.32mmol/g, 780mg). Standard double couple cycles were used throughout, except for <sup>8</sup>Arg-<sup>13</sup>D.Val which were given extended coupling times. Cys was protected with S<sup>t</sup>Bu groups. The final Fmoc group was left on the peptide, but the deprotection profile of <sup>2</sup>Val showed 99% coupling efficiency at this residue (yield peptide-resin 1.558g).

Fmoc peptide-resin (470mg) was sonicated in 20% piperidine/DMF (10ml) for 20 minutes to remove the Fmoc group, filtered and washed with DMF, 1,4-dioxan and



DMF. The peptide was deprotected and cleaved from the resin by stirring in TFA (10ml) with water (1ml), anisole (500 $\mu$ l), thioanisole (600 $\mu$ l) and EDT (200 $\mu$ l) under nitrogen for 2.5 hours. The crude peptide (152mg) was purified by preparative HPLC (column G; 5ml/min; (t,%B) (0,10)(10,40)(35,80)(36,90)(38,90)(40,10)) and lyophilised to give S' Bu protected peptide (40mg).

For removal of the cysteine protection, peptide (40mg) was dissolved in 5%water/TFE (20ml) and tributylphosphine (50 $\mu$ l) was added. The reaction was followed by analytical HPLC (column A) and after 2.5 hours most of the solvent was removed *in vacuo*. The peptide was precipitated by the addition of ether, filtered and washed with further ether.

Air oxidation was carried out immediately. The peptide was dissolved in tris buffer (0.1M, 400ml, pH8) and stirred vigorously for 42 hours. Once again, the reaction was followed by analytical HPLC (column A). The solution was lyophilised in two portions. The peptide was then separated from the salt by preparative HPLC (column G; 5ml/min; gradient (t,%B) (0,10)(5,30)(17,45)(19,90)(21,10)). An Ellman's test of the lyophilised material showed that it contained no free thiols.

FAB MS:  $MH^+$  2022.0 (for  $C_{90}H_{137}N_{23}O_{24}S_3$  expect 2022.4);

HPLC: (column D; gradient I; loop 100 $\mu$ l)  $R_t$  14.2 min;

AAA: Asp<sub>1</sub> 1.01, Ser<sub>2</sub> 1.56, Pro<sub>2</sub> 1.46, Ala<sub>1</sub> 1.10, Cys<sub>2</sub> 1.89, Val<sub>4</sub> 3.82, Met<sub>1</sub> 1.09, Leu<sub>1</sub> 1.16, Tyr<sub>1</sub> 0.89, Phe<sub>1</sub> 0.99, His<sub>1</sub> 0.93, Arg<sub>1</sub> 1.19



CD:	$\alpha$ -helix	$\beta$ -sheet	random coil
50mM sodium borate			
buffer	0%	54%	46%
50% TFE/sodium			
borate buffer	0%	46%	54%

**(+)-*cis*-aminocyclopentane-1-carboxylic acid**

(+)-*cis*-aminocyclopent-4-ene-1-carboxylic acid (499.6mg, 3.97mmol) was dissolved in methanol (70ml) and Pd/C, (50mg) was added. The mixture was stirred under hydrogen overnight.

The Pd/C was removed by filtration through celite and the methanol removed *in vacuo* to give the *title compound* as a white solid (yield 504.4mg, 98.5%).

mp: 200°C (dec); EI MS:  $MH^+$  129 (for  $C_6H_{11}NO_2$  expect 130); (found: C, 55.03; H, 8.84; N, 10.61, calc. for  $C_6H_{11}NO_2$ : C, 55.81; H, 8.53; N, 10.85%); FT IR (bromoform mull):  $\nu$  3018, 1640, 1542, 1400 $cm^{-1}$ ;  $^1H$  NMR (DMSO- $d_6$ /TFA- $d$ , 200MHz): 3.67, m, 1H; 2.81, m, 1H; 1.98-1.55, complex, 6H;  $[\alpha]_{589}^{23}$ : +10.0° (c, 1 in MeOH);  $R_f$  ( $CHCl_3$ :MeOH:AcOH, 85:10:5): 0 (baseline);  $R_f$  (nBuOH:py:H<sub>2</sub>O:AcOH, 15:10:12:3): 0.31.



**(+)-N-(9-fluorenylmethoxycarbonyl)-cis-3-aminocyclopentane-1-carboxylic acid**

(+)-Cis-3-aminocyclopentane-1-carboxylic acid (473mg, 3.67mmol) was suspended in water (10ml) and dioxan (20ml). Triethylamine (767 $\mu$ l, 5.5mmol, 1.5 equivalents) and 9-fluorenylmethyl succinimidyl carbonate (1.112g, 3.3mmol, 0.9 equivalents) were added and the resulting mixture was stirred at room temperature overnight.

The reaction mixture was diluted with water (100ml) and cooled on ice before being acidified to pH1.5 by the addition of 2M HCl. It was extracted with ethyl acetate (2x130ml), and the combined organic fractions were washed with water (120ml) and brine (120ml) before being dried over MgSO<sub>4</sub>.

The drying agent was removed by filtration, and the solvent was removed *in vacuo* to give the crude product. This was recrystallised from ethyl acetate/hexane to give the *title compound* as a white solid (yield 831.6mg, 71.8%).

mp: 166-167.5°C; FAB MS: MH<sup>+</sup> 352.15522 (for C<sub>21</sub>H<sub>22</sub>NO<sub>4</sub> expect 352.15488); (found: C, 71.86; H, 6.40; N, 3.82, calc. for C<sub>21</sub>H<sub>22</sub>NO<sub>4</sub>: C, 71.79, H, 5.98, N, 3.99); FT IR (bromoform mull):  $\nu$  3298, 3251, 3018, 1711, 1645, 1425cm<sup>-1</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 360MHz): 7.76-7.28, m, 8H; 4.46-4.11, m, 4H; 2.9, m, 1H; 2.26-1.69, complex, 6H; <sup>13</sup>C NMR (CDCl<sub>3</sub>, 90MHz): 181.912 (CO<sub>2</sub>H), 155.732 (urethane), 143.808, 141.195 (Fmoc Ar quarternary), 127.551, 126.913, 124.950, 119.835 (Fmoc Ar CH), 66.544 (Fmoc CH<sub>2</sub>), 52.479 (CH adjacent to NH), 47.120 (Fmoc CH), 41.559 (CH adjacent to CO<sub>2</sub>H), 39.952, 32.982, 27.932 (CH<sub>2</sub>); [ $\alpha$ ]<sub>D</sub><sup>23</sup><sub>589</sub>: +13.0° (c, 1



in MeOH);  $R_f$  (CHCl<sub>3</sub>:MeOH:AcOH, 85:10:5): 0.52;  $R_f$  (nBuOH:py:H<sub>2</sub>O:AcOH, 15:10:12:3): 0.75.

### **KU3 proIL-1 $\beta$ 113-122- $\beta$ -turn-mimic-160-163**

**Tyr Val His Asp Ala Pro Val Arg Ser Leu ACPC Met Ser Phe Val**

The synthesis was carried out on a 0.25 mmole scale, starting with FmocVal-resin (0.6mmol/g, 420mg). The first 5 residues, <sup>10</sup>Leu-<sup>14</sup>Phe, were coupled manually using bubbler apparatus. A Kaiser test was performed after each coupling, and the synthesis did not proceed to the next residue until no blue coloration was seen. The coupling methods and times were as follows: **Phe**, symmetrical anhydride (SA), 2 equivalents, 1 hour & HOBt activated ester (AE), 2 equivalents, 1.5 hours; **Ser**, SA, 2 equivalents, 1 hour; **Met**, SA, 2 equivalents, 1 hour & HOBt AE, 2 equivalents, 1.5 hours; **ACPC**, HOCT AE, 4 equivalents, overnight; **Leu** SA, 2 equivalents, 1 hour & HOBt AE, 2 equivalents, 2.25 and 1.25 hours. The synthesis was completed using automated double couple cycles. The overall coupling efficiency of the synthesis could not be ascertained due to the presence of an air bubble in the flow cell of the monitoring system (yield peptide-resin 843mg).

Deprotection and cleavage from the resin were carried out by stirring resin (400mg) in TFA (12ml) with water (2ml) and EDT (200 $\mu$ l) under nitrogen for 3 hours. The crude peptide (172mg) was purified by preparative HPLC (column G; 5ml/min;



gradient (t,%B) (0,10)(5,10)(30,50)(32,10)) and lyophilised to give the *title compound* (88mg).

FAB MS:  $MH^+$  1733.5 (for  $C_{80}H_{122}N_{20}O_{21}S$  expect 1733);

HPLC: (column A; gradient II; loop 2ml)  $R_t$  14mins;

AAA: Asp<sub>1</sub> 0.98, Ser<sub>2</sub> 1.71, Pro<sub>1</sub> 1.58, Ala<sub>1</sub> 1.12, Val<sub>3</sub> 3.14, Met<sub>1</sub> 1.06, Leu<sub>1</sub> 1.08, Tyr<sub>1</sub> 1.07, Phe<sub>1</sub> 0.83, His<sub>1</sub> 1.02, Arg<sub>1</sub> 1.10.

#### **KU4 proIL-1 $\beta$ 113-120**

##### **Tyr Val His Asp Ala Pro Val Arg**

The synthesis was carried out on a 0.25 mmole scale, starting with FmocArg(Pmc)-resin (0.59mmol/g, 425mg), standard double couple cycles being used throughout. The coupling efficiency for the final residue was 91.5% (yield peptide-resin 609mg).

Deprotection and cleavage were carried out by stirring peptide-resin (122mg) in TFA (5ml) with water (0.5ml) and anisole (0.25ml) under nitrogen for 3 hours. Crude peptide (41mg) was purified by preparative HPLC (column G; 5ml/min; gradient (t,%B) (0,10)(15,50)(17,10)) to give the *title compound* on lyophilisation (yield 21.6mg).

FAB MS:  $MH^+$  957 (for  $C_{43}H_{65}N_{13}O_{12}$  expect 957.1);



HPLC: (column C; gradient I; loop 2ml)  $R_t$  5min;

AAA: Asp<sub>1</sub> 1.02, Pro<sub>1</sub> 1.01, Ala<sub>1</sub> 1.12, Val<sub>2</sub> 1.98, Tyr<sub>1</sub> 0.96, His<sub>1</sub> 0.94, Arg<sub>1</sub> 0.97

**KU5 proIL-1 $\beta$  111-122 <sup>116</sup>Glu**

**Glu Ala Tyr Val His Glu Ala Pro Val Arg Ser Leu**

The synthesis was carried out on a 0.25mmole scale, starting with FmocLeu-resin (0.62mmol/g, 400mg) and using standard double couple cycles throughout. The overall coupling efficiency was 45% (yield peptide-resin 772mg).

Deprotection and cleavage were carried out by stirring peptide-resin (300mg) in TFA (10ml) with water (1.5ml) and anisole (0.5ml) for 3 hours. The crude peptide (130mg) was purified by preparative HPLC (column G; 5ml/min; gradient (t,%B) (0,10)(10,50)(12,10)) to give the *title compound* (yield 105mg).

FAB MS: MH<sup>+</sup> 1370.6 (for C<sub>61</sub>H<sub>95</sub>N<sub>17</sub>O<sub>19</sub> expect 1371.6)

HPLC: (column A; gradient I; loop 2ml)  $R_t$  9.2min

AAA: Ser<sub>1</sub> 0.92, Glu<sub>2</sub> 2.33, Pro<sub>1</sub> 1.03, Ala<sub>2</sub> 2.25, Val<sub>2</sub> 1.81, Leu<sub>1</sub> 1.06, Tyr<sub>1</sub> 0.99, His<sub>1</sub> 0.95, Arg<sub>1</sub> 1.01.



**KU6 proIL-1 $\beta$  111-122 <sup>119</sup>D.Val****Glu Ala Tyr Val His Asp Ala Pro D.Val Arg Ser Leu**

The synthesis was carried out on a 0.19mmole scale, starting with FmocLeu-resin (0.62mmol/g, 300mg). Double couple cycles were used throughout with <sup>1</sup>Glu to <sup>5</sup>His being given extended coupling times. The overall coupling efficiency was 90% (yield peptide-resin 646mg).

Deprotection and cleavage were carried out by stirring peptide-resin (296mg) in TFA (10ml) with water (1.5ml) and anisole (0.5ml) for 3 hours. The crude peptide (121mg) was purified by preparative HPLC (column G; 5ml/min; gradient (t,%B) (0,10)(15,50)(17,10)) to give the *title compound* (yield 90mg).

FAB MS: MH<sup>+</sup> 1356.4 (for C<sub>60</sub>H<sub>93</sub>N<sub>17</sub>O<sub>19</sub> expect 1357.5);

HPLC: (column A; gradient I; loop 2ml) R<sub>t</sub> 9.8min;

AAA: Asp<sub>1</sub> 1.03, Ser<sub>1</sub> 0.89, Glu<sub>1</sub> 1.16, Pro<sub>1</sub> 1.15, Ala<sub>2</sub> 2.10, Val<sub>2</sub> 1.76, Leu<sub>1</sub> 1.02, Tyr<sub>1</sub> 0.94, His<sub>1</sub> 0.91, Arg<sub>1</sub> 1.12.

**KU7 CrmA 300-307****Ac Leu Val Ala Asp Cys Ala Ser Thr NH<sub>2</sub>**

The synthesis was carried out on a 0.25mmole scale using Fmoc-amide resin (0.32mmo/g, 780mg). Standard double couple cycles were used throughout and the



cysteine was protected with a trityl group. The overall coupling efficiency of the synthesis was not ascertained due to the presence of an air bubble in the flow cell of the monitoring system. However, amino acid analysis of the crude, cleaved peptide indicated that approximately 50% was the required peptide. (Yield peptide-resin 1.097g).

Deprotection and cleavage were carried out by stirring peptide-resin (420mg) in TFA (10ml) with water (500μl), anisole (400μl) and EDT (100μl) under nitrogen for 1.5hours. The work-up was carried out using ether containing DTT (2%) and lyophilisation from 50mM ammonium acetate containing DTT (5%) gave the crude peptide (yield 142mg). The peptide was dissolved in aqueous 6M guanidine hydrochloride brought to a pH of approximately 9 by the addition of tris base, and containing DTT (2%) for preparative HPLC (column K; 9ml/min; gradient (t,%B) (0,10)(10,10)(55,90)(57,10)). Two major fractions were collected, of which the first was the *title compound* (yield 29.8mg).

FAB MS:  $MH^+$  820 (for  $C_{33}H_{57}N_9O_{13}S_1$  expect 820.9;

HPLC: (column B; gradient I; loop 2ml)  $R_t$  7min;

AAA: Asp<sub>1</sub> 1.01, Thr<sub>1</sub> 0.97, Ser<sub>1</sub> 0.91, Ala<sub>2</sub> 2.12, Cys<sub>1</sub> 0.63, Val<sub>1</sub> 1.05, Leu<sub>1</sub> 0.99.



**KU8 CrmA 300-307 <sup>304</sup>Ala<sup>305</sup>Cys****Ac Leu Val Ala Asp Ala Cys Ser Thr NH<sub>2</sub>**

The synthesis was carried out on a 0.25mmole scale using Fmoc-amide-resin (0.32mmol/g, 780mg). The cysteine was protected with a trityl group. <sup>6</sup>Cys-<sup>8</sup>Thr were coupled using standard double couple cycles. The remaining residues were all given extended coupling times with <sup>1</sup>Leu and <sup>5</sup>Ala being triple coupled (1xSA, 2xHOBt AE). The synthesis was interrupted before the final Fmoc deprotection and a small amount of resin removed so that the coupling efficiency could be checked manually (see 3.2.2). This showed an overall coupling efficiency of just 57%. Therefore the leucine was recoupled using the HOCT activated ester (no improvement in coupling efficiency was seen). Finally the leucine was recoupled using 4 equivalents of the HOBt activated ester with overnight sonication. This resulted in a final coupling efficiency of >90% (yield peptide-resin 1.068g).

Deprotection and cleavage were carried out by stirring peptide-resin (350mg) in TFA (10ml) with water (0.5ml), anisole (0.5ml) and EDT (100μl) under nitrogen for 2 hours (yield crude peptide 134mg). Work-up and preparative HPLC were as for KU7, giving two major fractions, of which the earlier eluting gave the *title compound* on lyophilisation (yield 11mg).

FAB MS: MH<sup>+</sup> 820 (for C<sub>33</sub>H<sub>57</sub>N<sub>9</sub>O<sub>13</sub>S<sub>1</sub> expect 820.9);

HPLC: (column A; gradient I; loop 2ml) R<sub>t</sub> 11.4 min;

AAA: Asp<sub>1</sub> 1.10, Ser<sub>1</sub> 0.87, Thr<sub>1</sub> 0.96, Ala<sub>2</sub> 2.21, Cys<sub>1</sub> 0.54, Val<sub>1</sub> 1.05, Leu<sub>1</sub> 1.00.



**Ac Cys Ala Leu Val Ala Asp Cys Ala Ser Thr Val Thr NH<sub>2</sub>**

The synthesis was carried out on a 0.25mmole scale, starting with Fmoc-amide-resin (0.32mmol/g, 780mg). Double couple cycles were used throughout, <sup>3</sup>Leu to <sup>6</sup>Asp being given extended coupling times. The cysteines were protected with Acm groups. The overall coupling efficiency was 87% (yield peptide-resin 1.239g).

Deprotection and cleavage were carried out by stirring peptide-resin (496mg) in TFA (10ml) with water (0.5ml) and anisole (0.5ml) under nitrogen for 2 hours 20 minutes. The crude peptide (197mg) was dissolved in basicified aqueous guanidine hydrochloride and purified by preparative HPLC (column K; 9ml/min; gradient (t, %B) (0,10)(10,10)(15,40)(35,50)(45,90)(47,10)). Lyophilisation gave the Acm protected peptide (139mg).

FAB MS: MH<sup>+</sup> 1336 (for C<sub>54</sub>H<sub>93</sub>N<sub>15</sub>O<sub>20</sub>S<sub>2</sub> expect 1337.6);

HPLC: (column D; gradient I; loop 5ml) R<sub>t</sub> 15.7min.

Removal of the Acm protecting groups was carried out using silylated glassware. Peptide (15mg) and silver trifluoromethanesulphonate (silver triflate) (20 equivalents, 0.224mmol, 58mg) were cooled on ice, and TFA (5ml) was added. The mixture was stirred in the dark with cooling on ice for 3 hours, after which most of the TFA was removed *in vacuo*. Care was taken to avoid taking the flask to dryness.



DTT (100 equivalents, 1.12mmol, 170mg) in tris buffer (0.1M, pH8, 5ml) was added to the flask and stirred in the dark, overnight, at room temperature. The suspension was transferred to a centrifuge tube and centrifuged at 4500rpm for 30 minutes. The supernatant was removed, the pellet resuspended in basicified aqueous 6M guanidine HCl and recentrifuged. The combined supernatants were purified by preparative HPLC (column F; 5ml/min; gradient (t,%B) (0,10)(25,35)(27,10)). Lyophilisation gave the *title compound* (yield 7.2mg) as a fluffy white solid giving a positive result in the Ellman's test.

FAB MS:  $MH^+$  1195 (for  $C_{48}H_{83}N_{13}O_{18}S_2$  expect 1195.4);

HPLC: (column C; gradient I; loop 2ml)  $R_t$  13.9 mins;

AAA: Asp<sub>1</sub> 1.25, Thr<sub>2</sub> 1.88, Ser<sub>1</sub> 0.86, Ala<sub>3</sub> 3.07, Cys<sub>2</sub> 0.37, Val<sub>2</sub> 2.01, Leu<sub>1</sub> 1.01.

Cyclisation was carried out by air oxidation. Peptide (10mg) was dissolved in aqueous ammonium acetate (50mM, pH9, 100ml) and stirred vigorously for 5 days. The mixture was lyophilised, redissolved in water and relyophilised to remove most of the ammonium acetate, before the last of the salt was removed by preparative HPLC (column F; 5ml/min; gradient (t,%B) (0,10)(15,40)(17,90)(19,10)). Lyophilisation gave the oxidised *title compound* (yield 3.2mg) as a fluffy, white solid giving a negative result in the Ellman's test.

FAB MS:  $MH^+$  1192 (for  $C_{48}H_{81}N_{13}O_{18}S_2$  expect 1193.4);

HPLC: (column D; gradient I; loop 2ml)  $R_t$  13.6min;



AAA: Asp<sub>1</sub> 1.09, Thr<sub>2</sub> 1.83, Ser<sub>1</sub> 1.02, Ala<sub>3</sub> 3.00, Cys<sub>2</sub> 1.60, Val<sub>2</sub> 2.00, Leu<sub>1</sub> 0.98

**KU11 ICE pro region 89-119**

**Leu Ser Ala Asp Gln Thr Ser Gly Asn Tyr Leu Asn Met Gln Asp Ser Gln Gly  
Val Leu Ser Ser Phe Pro Ala Pro Gln Ala Val Gln Asp**

The synthesis was carried out on a 0.25mmole scale, starting with Fmoc-Asp(O<sup>t</sup>Bu)-resin (0.664mmol/g, 377mg). Asn and Gln were coupled without side-chain protection. Standard double couple cycles were used throughout and the final Fmoc was left on the peptide. The overall coupling efficiency was 50% (yield Fmoc-peptide-resin 1.292g).

Fmoc-peptide-resin (130mg) was sonicated in 20% piperidine/DMF (5ml) for 5 minutes, filtered and washed with DMF, dioxan and ether. Deprotection and cleavage were carried out by stirring the peptide-resin in TFA (5ml) with water (0.5ml), anisole (200μl) and EDT (100μl) under nitrogen for 3 hours. The crude peptide (65mg) was purified by preparative HPLC (column J; 5ml/min; gradient (t,%B) (0,10)(35,40)(37,90)(39,10)). Lyophilisation gave the *title compound* (yield 18.1mg).

FAB MS: MH<sup>+</sup> 3268.9 (for C<sub>138</sub>H<sub>215</sub>N<sub>38</sub>O<sub>52</sub>S<sub>1</sub> expect 3270.5);

HPLC: (column A; gradient I; loop 5ml) R<sub>t</sub> 16.7min;



AAA: Asp<sub>5</sub> 5.34, Thr<sub>1</sub> 0.91, Ser<sub>5</sub> 5.13, Glu<sub>5</sub> 6.64, Pro<sub>2</sub> 2.97, Gly<sub>2</sub> 2.18, Ala<sub>3</sub> 3.28, Val<sub>2</sub> 2.13, Met<sub>1</sub> 0.79, Leu<sub>3</sub> 2.85, Tyr<sub>1</sub> 1.00, Phe<sub>1</sub> 1.05.

### **Dibenzocycloheptadiene-5-one methoxycarboxy-Ala-PAM Resin**

Boc-Ala-PAM resin (2.86g, 0.56mmol/g, 1.5mmol) was swollen with DCM (5ml). TFA (5ml) was added and the resin was sonicated for 10 minutes. The resin was filtered and washed with copious DCM, 10%DIEA/DMF and DCM.

2-(Oxyacetic acid)dibenzocycloheptadiene-5-one (846mg, 3mmol) was placed in a round-bottomed flask with HOBt (0.25M solution in DMF, 12ml, 3mmol). DIC (0.25M solution in dioxan, 12ml, 3mmol) was added and the mixture stirred for 15 minutes. This was then added to dry Ala-PAM resin and the suspension was stirred mechanically overnight.

The resin was filtered and washed with DMF, methanol, further DMF, and dried with ether (yield 2.92g). A quantitative Kaiser test showed that coupling had proceeded 99.9%.

FT IR (KBr disc):  $\nu$  1738, 1685, 1640, 1597cm<sup>-1</sup>.



### **Dibenzocycloheptadiene-5-ol methoxycarboxy-Ala-PAM resin**

Dibenzocycloheptadiene-5-one methoxycarboxy-Ala-PAM resin (2.92g), 0.51mmol/g, 1.5mmol) was swollen with THF (Na dried, 40ml) and lithium borohydride (33mg, 1.5mmole) was added. The mixture was stirred mechanically at room temperature for 1 hour.

The resin was filtered and washed with copious methanol, 0.001M HCl, methanol, and dried with ether (yield 2.90g).

FT IR (KBr disc):  $\nu$  1740, 1685, 1600 $\text{cm}^{-1}$ .

### **N -*t*- Butyloxycarbonyl -N'- dibenzocycloheptadiene -5- yl methoxycarboxy-Ala-PAM resin hydrazine (Boc-hydrazide Resin)**

Dibenzocycloheptadiene-5-ol methoxy carboxy-Ala-PAM resin (2.9g) was placed in a round-bottomed flask with t-butylcarbazate (1.386g, 10.5mmol). DCM was added, followed by benzenesulphonic acid (118mg, 0.75mmol), and the mixture heated at reflux, with mechanical stirring, for 6 hours. The resin was filtered and washed with copious DCM (yield 2.95g).

FT IR (KBr disc):  $\nu$  1740, 1685, 1600 $\text{cm}^{-1}$ .



### **FmocGly-hydrazide Resin**

FmocGlyOH (1.458g, 5mmol) was suspended in DCM (10ml) and oxalyl chloride (880 $\mu$ l, 10mmol) was added, followed by DMF (2 drops). The mixture was stirred for 1 hour, until the reaction was complete. The DCM and excess oxalyl chloride were removed *in vacuo*. The residue was redissolved in DCM (10ml) and added to Boc-hydrazide resin (500mg), preswollen in DCM (15ml) and pyridine (5ml). The mixture was heated under reflux for 4.5 hours.

The resin was filtered and washed with copious DCM (yield 533mg). An Fmoc loading test revealed an Fmoc-Gly loading of 0.25mmol/g.

### **KU13 Leu Ile Phe Ala Gly NHNH<sub>2</sub>**

The synthesis was carried out on a 0.13mmole scale, starting with Fmoc-Gly-hydrazide resin (0.25mmol/g, 530mg). The synthesis was carried out manually using bubbler apparatus; a Kaiser test was performed after each coupling and the synthesis did not proceed to the next residue until no blue coloration was seen. Unless otherwise stated, each coupling used 4 equivalents of the relevant activated amino acid species. The coupling times and methods were as follows: **Ala**, symmetrical anhydride (SA), 1 hour 20 minutes; **Phe**, SA, 1 hour 10 mins & HOBt activated ester



(AE), 45 mins; **Ile**, SA, 35 mins & AE, over night; **Leu**, SA, 8 equivalents, 3 hours.  
(Yield peptide-resin 540mg).

Cleavage was carried out by stirring the peptide-resin in 90% TFA/H<sub>2</sub>O (5ml) under nitrogen for 1.5 hours. The crude peptide (42mg) was shown by analytical HPLC to contain few impurities. Since the purpose of the synthesis was to check the utility of the resin, not to obtain a molecule for biological testing, the peptide was not purified.

FAB MS: MH<sup>+</sup> 534 (for C<sub>26</sub>H<sub>44</sub>N<sub>7</sub>O<sub>5</sub> expect 534.7);

HPLC: (column D; gradient I; loop 2ml) R<sub>t</sub> 11.7 min.

#### **KU14b ICE pro region 1-119**

The synthesis was carried out on a 0.1mmole scale, starting with FmocAsp(O<sup>t</sup>Bu)-resin (0.16mmol/g, 635mg). The Asn and Gln residues were protected by Mbh groups, and the Cys residues by MeOPhenac. Standard double couple cycles for large peptides were used throughout, except in four regions, <sup>12</sup>L-<sup>15</sup>R, <sup>44</sup>K-<sup>49</sup>T, <sup>58</sup>I-<sup>62</sup>I and <sup>71</sup>I-<sup>76</sup>I, where extended coupling times were used. Approximately one third of the peptide-resin (627mg dry weight) was removed from the reaction vessel after coupling of <sup>63</sup>P. The final Fmoc was left on the resin (yield Fmoc-peptide-resin 1.31g). From the deprotection profile, and an Fmoc loading test of the completed peptide, it was estimated that the overall coupling efficiency was approximately 40%.



The final Fmoc was removed by sonicating the peptide-resin in 20% piperidine/DMF for 15 minutes, followed by filtration and washing with DMF, dioxan, DMF, and drying with ether. TbFmoc was loaded using standard methodology (see section 3.2.6).

Deprotection and cleavage from the resin (118mg) was carried out using the TMSBr cleavage procedure (see section 3.2.8) for 6 hours. Lyophilisation gave crude TbFmoc-peptide as a fluffy, white solid (60mg). Crude TbFmoc-peptide (18mg) was dissolved in 70% acetonitrile/water (7ml) and stirred with porous graphitised carbon (PGC) (350mg) for 15 minutes. The suspension was filtered through a layer of celite in a 1cm diameter, straight-sided sinter funnel. The PGC column was washed with 70% acetonitrile/water (15ml) and 50% *iso*-propanol/6M guanidine HCl (15ml), both washing solutions being passed through the column twice. The TbFmoc group was cleaved from the peptide, and the peptide washed from the column, by passing a 10% solution of piperidine in 50% *iso*-propanol/6M guanidine HCl (20ml) through the column twice.

The deprotection filtrate was neutralised by the addition of acetic acid (2ml) and the *iso*-propanol removed *in vacuo*. The peptide solution was desalted by gel filtration through a 10mm x 75cm G50 Sephadex gel column, eluted with 30% acetic acid/water, running at 20ml/hour, 30 minute fractions being collected. The relevant fractions (5, 6 & 7) were combined and lyophilised to give cysteine-protected peptide (5mg).



Removal of the cysteine protecting groups was carried out using mercuric acetate. Peptide (16.3mg, approx.  $1.19 \times 10^{-3}$  mmol) was dissolved/suspended in 50% acetic acid/water (10ml), and mercuric acetate (6mg,  $1.43 \times 10^{-2}$  mmol, 16 equivalents) was added. The reaction mixture was stirred overnight, after which  $\beta$ -mercaptoethanol (BME) (0.5ml) was added and the mixture stirred overnight once more. The  $\text{Hg}^{\text{II}}$ -BME complex, which formed a fine suspension, was removed by centrifugation and the supernatant was subjected to gel filtration as before (yield 5mg). A quantitative Ellman's test gave an average of 1.8 free thiols per molecule.

AAA: Asp<sub>13</sub> 11.37, Thr<sub>7</sub> 6.53, Ser<sub>8</sub> 5.57, Glu<sub>17</sub> 18.30, Pro<sub>3</sub> 1.66, Gly<sub>8</sub> 7.66, Ala<sub>9</sub> 8.90, Cys<sub>3</sub> 2.48, Val<sub>7</sub> 7.47, Met<sub>5</sub> 6.15, Ile<sub>7</sub> 7.53, Leu<sub>13</sub> 12.22, Tyr<sub>3</sub> 1.18, Phe<sub>2</sub> 1.91, Lys<sub>9</sub> 8.07, Arg<sub>5</sub> 4.28.

#### **KU17 HIV<sub>IIIB</sub> gp120 Lys 364-378 ProD.Val 445-459**

**Lys Ser Ser Gly Gly Asp Pro Glu Ile Val Thr His Ser Phe Asn Cys Pro D.Val  
Cys Ser Ser Asn Ile Thr Gly Leu Leu Leu Thr Arg Asp Gly Gly**

The synthesis was carried out on a 0.25 mmole scale using Fmoc-Gly-resin (0.615mmol/g, 406mg). Double couple cycles were used throughout,  $^{10}\text{Val}$ - $^{21}\text{Ser}$  being given extended coupling times. Cys was protected with S<sup>t</sup>Bu groups. Asn was coupled without side-chain protection. The efficiency of the final coupling was 65% (yield Fmoc-peptide-resin 1.348g).



Fmoc-peptide-resin (370mg) was sonicated with 20% piperidine/DMF for 10 minutes, filtered and washed successively with DMF, dioxan and ether. The peptide was deprotected and cleaved from the resin by stirring with TFA (9ml), water (1ml) and anisole (0.5ml) under nitrogen for 3 hours. The crude peptide (249mg) was purified by preparative HPLC (column J; 5ml/min; gradient (t, %B) (0,30)(18,45)(20,90)(22,30)) to give the cysteine-protected peptide (45mg).

TOF MS:  $MH^+$  3538.8 (for  $C_{149}H_{246}N_{41}O_{50}S_4$  expect 3540.1);

HPLC: (column D; gradient I; loop 2ml)  $R_t$  16.7 min;

To remove the  $S^tBu$  protecting groups, peptide (55mg) was dissolved in 5% $H_2O$ /TFE (20ml) and tributylphosphine (50 $\mu$ l) was added. The mixture was stirred for 2 hours 40 minutes. Most of the solvent was removed *in vacuo* and the peptide was precipitated by the addition of ether. The peptide was filtered and washed with further ether, before being dissolved in aqueous acetic acid for lyophilisation (yield 53.5mg).

Air oxidation of the peptide was carried out by vigorous stirring in 50mM ammonium acetate buffer (pH 8, 500ml) for 7 days. Lyophilisation and preparative HPLC (column H; 5ml/min; gradient (t,%B) (0,10)(17,32)(19,90)(21,10)) gave the oxidised *title compound* (yield 8.5mg).

TOF MS:  $MH^+$  3359.5 (for  $C_{141}H_{228}N_{41}O_{50}S_2$  expect 3361.7);



HPLC: (column E; gradient I; loop 2ml) R<sub>t</sub> 15.4 min;

AAA: Asp<sub>4</sub> 4.07, Thr<sub>3</sub> 2.85, Ser<sub>5</sub> 4.37, Glu<sub>1</sub> 1.20, Pro<sub>2</sub> 2.16, Gly<sub>5</sub> 5.11, Cys<sub>2</sub> 1.74, Val<sub>2</sub> 1.93, Ile<sub>2</sub> 1.63, Leu<sub>3</sub> 2.93, Phe<sub>1</sub> 1.15, His<sub>1</sub> 0.97, Lys<sub>1</sub> 0.98, Arg<sub>1</sub> 0.96.

**KU18 HIV<sub>IIIB</sub> gp120 Lys 364-377 ACPC 446-459**

**Lys Ser Ser Gly Gly Asp Pro Glu Ile Val Thr His Ser Phe Asn ACPC Ser Ser  
Asn Ile Thr Gly Leu Leu Leu Thr Arg Asp Gly Gly**

The synthesis was carried out on a 0.2 mmole scale using Fmoc-Gly-resin (0.615 mmol/g, 325mg) in two stages. The synthesis proceeded as normal, employing standard double couple cycles, until <sup>17</sup>Ser, when it was interrupted. The resin was removed from the reaction vessel and added to the HOCT activated ester of FmocACPC, preformed by stirring FmocACPC (0.4mmol, 140mg) and HOCT (0.4mmol, 63mg) in the presence of DIC (0.4mmol, 64μl) in DMF/dioxan (1:1, 5ml) for 10 minutes. The reaction mixture was sonicated over night at 26°C. The resin was then filtered and washed, and returned to the reaction vessel for completion of the synthesis by standard methodology. The deprotection profile showed a significant drop of about 19% in coupling efficiency for the cyclopentyl residue. However this was probably, at least partly, due to mechanical losses associated with transferring the resin between reaction vessels. The overall coupling efficiency was 66% (yield peptide-resin 901.5mg).



Deprotection and cleavage were carried out by stirring resin (290mg) in TFA (10ml) with water (1ml), thioanisole (0.5ml) and anisole (0.5ml) under nitrogen for 3 hours. The crude peptide (200mg) was purified by preparative HPLC (column J; 5ml/min; gradient (t,%B) (0,10)(40,90)(42,10)) and lyophilised to give the *title compound* (13mg).

TOF MS:  $MH^+$  3072.9 (for  $C_{131}H_{213}N_{38}O_{47}$  expect 3072.4);

HPLC: (column E; gradient I; loop 5ml)  $R_t$  16.2min;

AAA: Asp<sub>4</sub> 4.59, Thr<sub>3</sub> 3.14, Ser<sub>5</sub> 4.65, Glu<sub>1</sub> 1.13, Pro<sub>1</sub> 1.17, Gly<sub>5</sub> 5.71, Val<sub>1</sub> 0.96, Ile<sub>2</sub> 1.83, Leu<sub>3</sub> 3.59, Phe<sub>1</sub> 1.03, His<sub>1</sub> 1.01, Lys<sub>1</sub> 1.01, Arg<sub>1</sub> 1.20;

CD:	$\alpha$ -helix	$\beta$ -sheet	random coil
50mM sodium borate			
buffer	0%	51%	48%
50% TFE/sodium			
borate buffer	11%	53%	37%

## Fluorescence Assay

The assay was run at 30°C. The assay buffer (100mM sodium HEPES, pH 7.5, 10mM DTT, 10% sucrose, 0.1% CHAPS, 3ml) was placed in a cuvette with the substrate, AcTyrValAlaAspAMC, (10mM in DMF, 15 $\mu$ l), and allowed to equilibrate to the assay temperature. A variable volume (15-60 $\mu$ l) of an aqueous solution (10-20mM) of the test peptide was added to the cuvette, followed by cell lysate



containing crude ICE (15 $\mu$ l, provided by Dr D Giegel, Parke-Davis Pharmaceuticals, Ann Arbor, MI). The cuvette was inverted 3 times to mix the contents and returned to the fluorimeter, when recording of the timed spectrum commenced immediately.

The change in fluorescence of the sample was followed over 15 minutes, a data point being recorded every second. The excitation wavelength used was 380nm and the emission 460nm; the slit width used for both was 10.0.

### **HPLC Assay**

Assay buffer (39 $\mu$ l), enzyme lysate (10 $\mu$ l) and test peptide solution (10mM in water, 1 $\mu$ l) were mixed in a 0.5ml Eppendorf tube and incubated at 30°C for variable times (1-24 hours). The enzyme mixture was injected onto a Vydac C<sub>18</sub> analytical column, and an increasing acetonitrile gradient run as follows: (t,%B) (0,5)(20,30)(22,5).

### **KU16 proIL-1 $\beta$ 117-122**

#### **Ala Pro Val Arg Ser Leu**

The synthesis was carried out on a 0.24mmole scale, starting with FmocLeu-resin (0.62mmol/g, 387mg) and using standard double couple cycles throughout. The overall coupling efficiency was 93% (yield peptide-resin 533mg).



Deprotection and cleavage were carried out by stirring peptide-resin (320mg) in TFA (10ml) with water (1ml) and anisole (200 $\mu$ l) under nitrogen for 1.5 hours. Lyophilisation gave a white fluffy solid which was shown to be a single product by analytical HPLC (yield 65mg).

FAB MS:  $MH^+$  643 (for  $C_{28}H_{51}N_9O_8$  expect 642.8);

HPLC: (column D; loop 2ml; gradient (t,%B) (0,5)(20,30)(22,5))  $R_t$  10.7 min;

AAA: Ser<sub>1</sub> 0.90, Pro<sub>1</sub> 1.02, Ala<sub>1</sub> 1.05, Val<sub>1</sub> 1.03, Leu<sub>1</sub> 0.99, Arg<sub>1</sub> 1.02.

### **KU15 proIL-1 $\beta$ 111-116**

#### **Glu Ala Tyr Val His Asp**

The synthesis was begun on a 0.25mmole scale, starting with FmocAsp(O<sup>t</sup>Bu)-resin (0.57mmol/g, 440mg). As approximately half of the resin was removed after the coupling of <sup>3</sup>Tyr, an accurate estimate of the coupling efficiency could not be made from the deprotection profile (yield peptide-resin 286mg).

Deprotection and cleavage were carried out as for KU16, analytical HPLC again showing a single product (yield 92mg).

FAB MS:  $MH^+$  734 (for  $C_{32}H_{44}N_8O_{12}$  expect 733.8);

HPLC: (conditions as for KU92)  $R_t$  8.0 min;

AAA: Asp<sub>1</sub> 0.99, Glu<sub>1</sub> 1.14; Ala<sub>1</sub> 1.03, Val<sub>1</sub> 0.96, Tyr<sub>1</sub> 0.98, His<sub>1</sub> 0.91.



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## **Courses & Conferences Attended**

Organic Research Seminars, various speakers, 1991-94

Royal Society of Chemistry Perkin Division Scottish Meeting, various speakers, Heriot-Watt 1991, Edinburgh 1992, Aberdeen 1993.

The Royal Society of Edinburgh Symposium on Protein Engineering, various speakers, Edinburgh, 1992

Medicinal Chemistry, Professor R Baker, Merck Sharp & Dohme, 1992-93

Chemical Development in the Pharmaceutical Industry, various speakers, SmithKline Beecham, 1993-94

NMR spectroscopy, Dr IH Sadler, Edinburgh University, 1993

Innovation & Perspectives in Solid Phase Synthesis, various speakers, Oxford, 31 Aug - 4 Sept 1993

New Horizons in Anti-Inflammatory Therapy, various speakers, London, 11 May 1994